Prion Protein Self-Interactions:

a gateway to novel therapeutic strategies?

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Prion Protein Self-Interactions:

a gateway to novel therapeutic strategies?

Interacties Tussen Prion Eiwitten:

de poort naar vernieuwende therapieën?

(met een samenvatting in het Nederlands)

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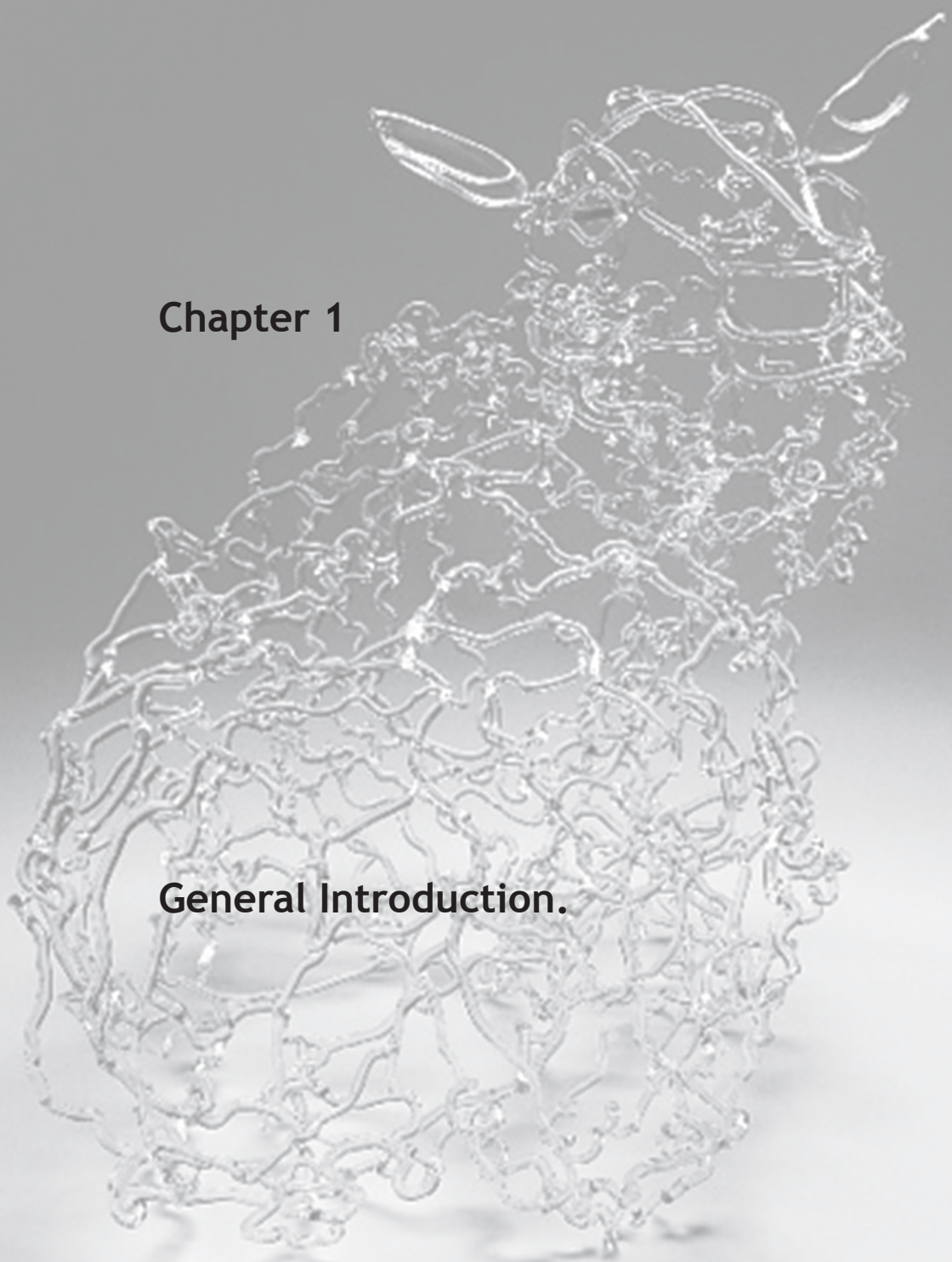
Contents.



Chapter 1

General Introduction.

Transmissible Spongiform Encephalopathies.

Transmissible Spongiform Encephalopathies (TSEs) or prion diseases are comprised of fatal neurodegenerative disorders affecting both human and animals. Prion diseases cause sponge-like degeneration of neuronal tissue and include (among others) Creutzfeldt-Jacob disease in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. TSEs are characterized by formation and accumulation of transmissible (infectious) disease associated protease resistant prion protein (PrP^{Sc}) mainly in tissues of the central nervous system.

Initially it was assumed that the causative agent was a virus, but the inability to isolate a virus or even detect an immunological response raised questions as to the nature of the causative agent. Especially when the transmissible agent of prion disease proved resistant to nucleic acids inactivating treatments (i.e. ultraviolet radiation or treatment with nucleases). These results resulted in speculations that the transmissible agent may be devoid of nucleic acid [1, 2]. Griffith however, was first to postulate the theory that the transmissible agent was comprised solely of protein [3]. He proposed three different hypothetical theories of propagation by protein only, one of which closely resembles current hypothesis. The term prion however, a derivative of proteinacious infectious particle, was first proposed by Prusiner in 1982 [4], to distinguish the infectious pathogen from viruses or viroids. Prions were defined as "small proteinacious particles that resist inactivation by procedures which modify nucleic acids".

Even though TSEs are rare disorders, in humans only affecting one or two persons out of each million people each year on a global scale, research into prion diseases has increased. This increase in research was boosted by the BSE (mad-cow disease, 1986) crisis in the UK as a result of feeding cattle protein supplements containing sheep and cattle offal. This resulted in cannibalizing of -and allowing increased replication of the infectious agent. These protein supplements were banned in 1988, but not until 1991-1992 this ban was strictly enforced. In 1996 the first cases of a new variant of Creutzfeldt-Jacob disease (nvCJD or vCJD) in humans were reported, now widely accepted as a result of consumption of BSE infected beef or beef products. The emergence of this new disease, combined with the long incubation period of TSEs sparked the fear of a vCJD epidemic of biblical proportions.

Veterinary and human TSEs.

Scrapie is a naturally occurring disease in sheep and goats (reviewed in [5]), prevalent in many countries worldwide and recognized in Europe for over 200 years, with the first case of scrapie (in the UK) described in 1732. It is considered the prototype of prion disease, especially because other TSEs in animals were only recognized over the last few decades, with BSE only since the 1980s. Transmission of scrapie is believed to be from ewe to lambs because of contact with the placenta or placental fluids. Development of the disease is a very slow process and varies widely among individual animals. However when clinical signs are apparent, the disease usually progresses rapidly. Usually scrapie infected animals show behavioural differences (i.e. tremors, individual separates itself from the flock) and overall itching making the animal scrape uncontrollably against posts and fences (hence the term scrapie, Fig.1) resulting in bald patches and scabs.

Figure 1. ***Scrapie sheep.*** *Example of a sheep in clinical phase of scrapie disease, showing result of continued (obsessive) scratching to relief the itching.*

In 1986 the first case of ***bovine spongiform encephalopathy*** (BSE) was reported in the UK, soon after growing into an epidemic with over 180.000 confirmed cases and mathematical models suggesting 1-3 million cattle infected (reviewed in [5]). It is now generally accepted that the epidemic is caused by the recycling of ruminant tissues contaminated with BSE in food supplements for cattle, with bone-meal as the main culprit. Onset of the disease is usually characterized by differences in behaviour such as the animal separating itself from the flock, heightened sensitivity to sound and other stimuli slowly getting progressively worse, resulting in the inability to handle the animal because it panics or even becomes aggressive. While the disease progresses, changes in movement can be observed ranging from a swaying ('drunken') gait, a broad stance combined with lifting the legs up high ending in difficulty in turning and falling down finally leading to death.

Creutzfeldt-Jacob disease (CJD) is a human TSE and divided in four types of CJD, sporadic (sCJD), familial (fCJD), iatrogenic (iCDJ) and variant (vCJD). The main variant of CJD is ***sporadic CJD*** (sCJD), which is a rare occurring disease, only one in a million people worldwide is affected (reviewed in [6]). Sporadic CJD occurs mainly in people of middle and old age, with a peak onset between 60-65 years of age. The disease unveils itself by symptoms as declining mental capabilities (i.e. loss of short term memory, dementia) or emotional disturbances together with loss of the fine motor skills. Characteristic for sCJD is the rapid progress of clinical symptoms; these clinical symptoms progress over weeks and can become fatal after only a few months. Definitive ascertainment of the diagnosis can only be done by post-mortem microscopic analysis of the brain and sCJD is determined by widespread spongiform vacuolisation and the accumulations of only a few or no amyloid plaques. The tissues frequently affected are the cerebral cortex, basal ganglia and caudate nuclei, and the cerebellum.

Familial CJD (fCJD) is (as suggested by the name) a hereditary form of CJD and is inherited in an autosomal dominant manner (reviewed in [6]). Familial CJD cases are either caused by a single amino acid mutation or by the insertion of extra octapeptide repeats. Both clinical and pathological symptoms of fCJD are comparable to those found in sCJD, but vary depending on the specific mutation present. Usually fCJD has an earlier onset and a longer duration when compared to sCJD.

Iatrogenic CJD (iCJD) can occur as a result of a medical procedure (reviewed in [6]) like organ donation, in which the patient has received donor material from a CJD affected person. Grafts of infected corneas and dura mater have been shown to transmit CJD. Brain surgery with instruments improperly sterilised have also been shown to be a source for iCJD. Another source of infection can also be treatment with cadaveric pituitary human growth hormone (HGH) or cadaveric pituitary gonadotropin. The incubation period for iCJD ranges from 1.5 to 18 years and onset of iCJD is mostly characterized by loss of coordination, whereas symptoms of dementia occur much later or even remain absent.

In 1996 however, 10 unusual cases of CJD were reported and because of these unusual characteristics named ***variant CJD*** (vCJD) [7]. These cases had in common that all patients were young, had no reported neuropathological changes in the brain, and unlike sCJD psychiatric features are the fisrt clinical features presented. Clinical and pathological features in macaques experimentally infected with brain homogenates from BSE-affected cows [8] and the biochemical properties of PrP^{Sc} associated with vCJD in humans and BSE show similarities. These similarities are the foundation for

the conclusion that ingestion of infected beef and beef products is the most likely cause of the emergence of vCJD. Several observations suggest that vCJD is a distinct novel disease, firstly due to the distinct pathology in vCJD characterized by abundant large amyloid plaques surrounded by a halo of spongiform changes [9] and secondly because of the significantly lower age of onset when compared to sCJD. Early clinical symptoms are psychiatric, usually in the form of depression or less often as schizophrenia-like psychosis. As the disease progresses neurological signs like unsteadiness, difficulty walking and involuntary movements develop and when the disease reaches near fatal conditions patients become completely immobile and mute.

Route of Infection.

Infectious prion diseases like sheep scrapie, BSE in cattle and vCJD in humans typically occur via the oral route. This route necessitates primary replication of the infectious agent in a peripheral compartment prior to brain invasion (reviewed in [10], Fig.2). After oral infection PrP^{Sc} can be identified in gut Peyer's patches and is followed by prion propagation to splenic lymphoid tissue and spleen and/or in gut-associated lymphoid tissue (GALT; including tonsil), resulting in prion transport by splenic innervation via the enteric nervous system (ENS) to the brainstem and spinal cord. Follicular Dendritic Cells (FDCs) seem to be of specific interest. These cells reside in the immune follicles of the gut, lymph nodes and spleen and acquire a large load of PrP^{Sc} during infection and therefore seem vital for propagation of infectivity in these tissues. Furthermore, FDCs are long-lived cells that express high levels of PrP^{C} (similar to neurons), and are specialized to trap, retain and present unprocessed antigens, which supports their role in the spread of prion infectivity. Especially the route of infection in sheep is very well documented, the progression (in time) defined from oral infection to the start of prion accumulation at the port of entry, the spread to the spinal column and finally to the brain of the sheep [11, 12], however the specific cells or route of spread are not completely clear. Basically (natural) prion infection of sheep can be broken down into three phases; the first phase after ingestion of the scrapie agent is invasion of the GALT, more precisely the GALT of the oropharynx and the gut.

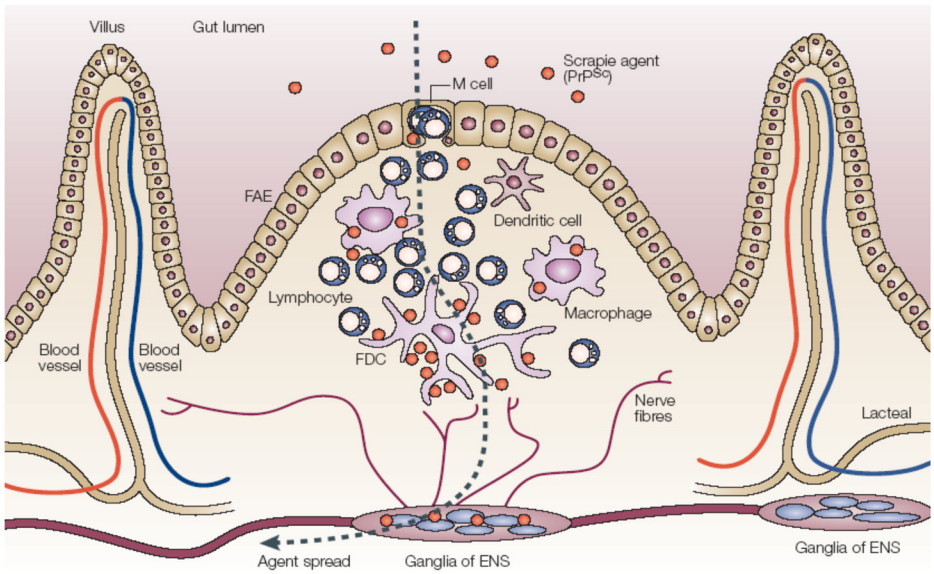
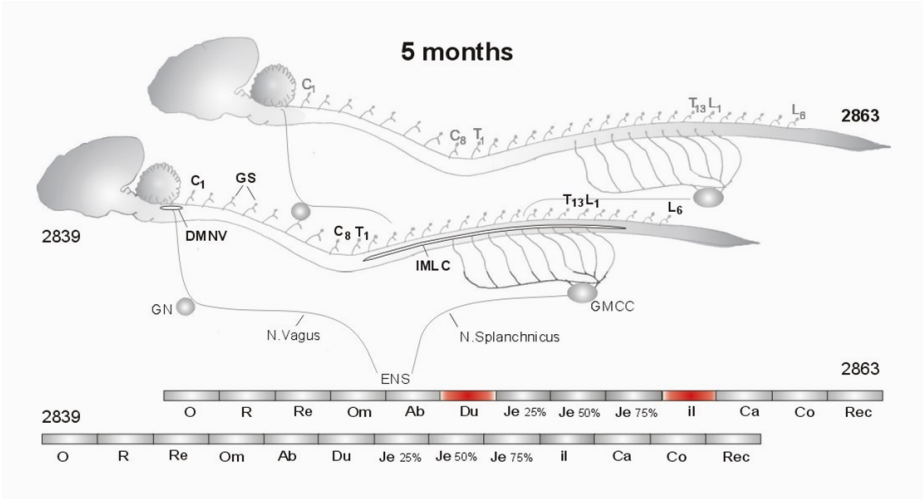
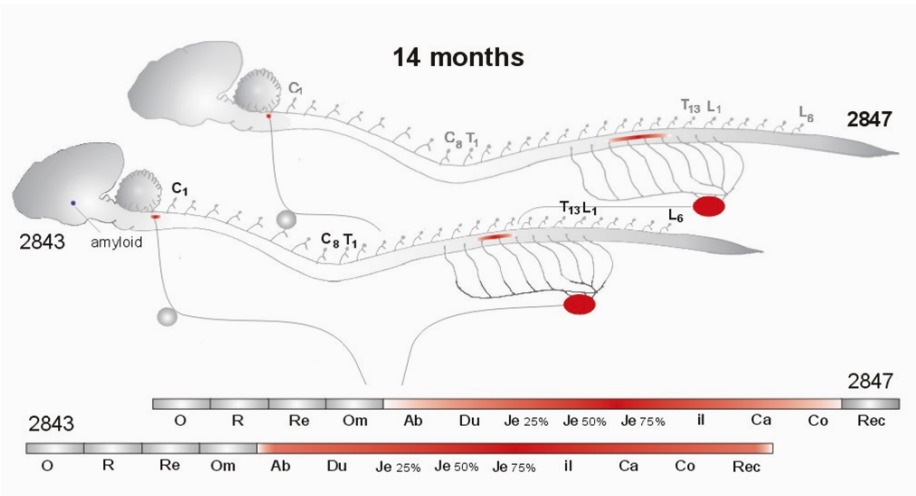
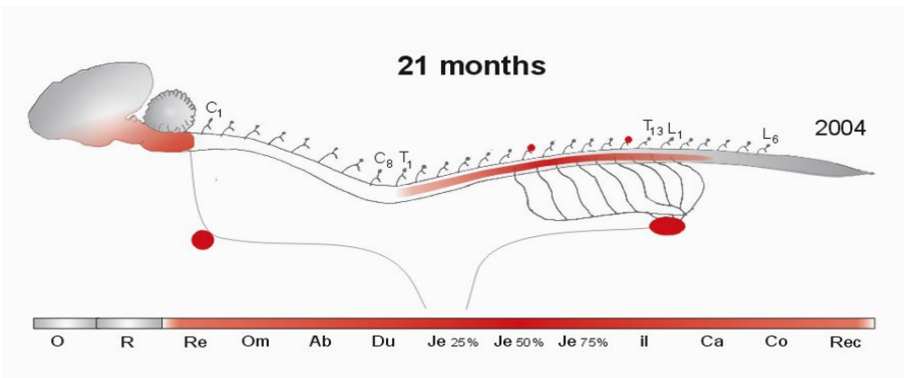


Figure 2. ***Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection (route indicated by dotted line).*** *Soon after ingestion, the abnormal prion isoform (PrP^{Sc}) is detected readily within Peyer's patches on follicular dendritic cells (FDCs), within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations indicate that, following uptake of scrapie infectivity from the gut lumen, infectivity accumulates on FDCs in Peyer's patches and subsequently spreads via the ENS to the central nervous system (reproduced from Cashman, N.R. and B. Caughey,* Prion diseases--close to effective therapy? *Nat Rev Drug Discov, 2004. 3(10): p. 874-84, adapted from [13] with permission from © Elsevier Ltd. [2000]).*

Phase two is accomplished when non-GALT lymphoid tissues are infected, when so called lymphatic dissemination has taken place. This is most likely due to PrP^{Sc} containing and free-ranging cells that were found within the cortical and paracortical sinuses, which gained access to the efferent lymph nodes and subsequently the blood stream. The third phase of scrapie pathogenesis is neuroinvasion (Fig.3), the route of infection likely due to infection of the ENS (closely related with Peyer's patches) followed by infection of the parasympathetic and sympathetic efferent neuronal pathways and finally infecting the brain and spinal cord (Fig.3). Other possible (theoretical) routes of infection of the Central Nervous System (CNS) are either spread through peripheral nerve endings originating from infected non-GALT lymphoid tissues or by haematogenic spread (more detailed description in [11, 12]).


5 months
C1
C8 T1
T13 L1
L6
2863
2839
GS
DMNV
IML C
GN
GMCC
N.Vagus
N.Splanchnicus
ENS
O R Re Om Ab Du Je 25% Je 50% Je 75% il Ca Co Rec


14 months
C1
C8 T1
T13 L1
L6
2847
2843
amyloid
O R Re Om Ab Du Je 25% Je 50% Je 75% il Ca Co Rec


21 months
C1
C8 T1
T13 L1
L6
2004
O R Re Om Ab Du Je 25% Je 50% Je 75% il Ca Co Rec

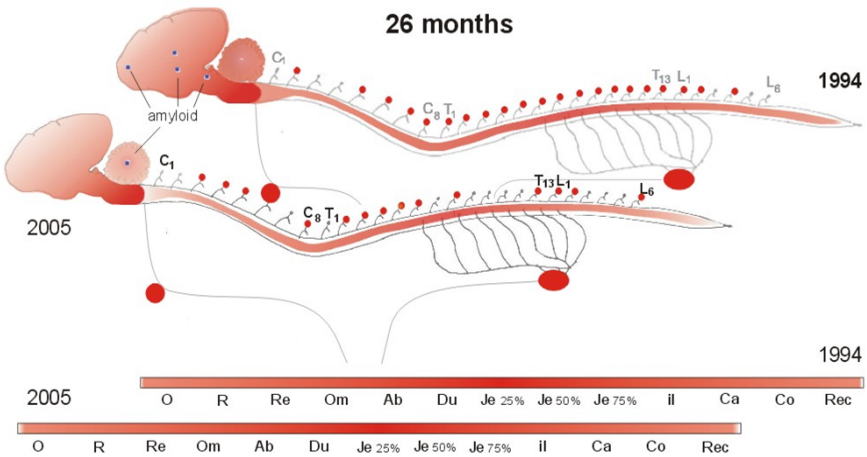


Figure 3. ***Schematic representation of PrP^{Sc} accumulation.*** *PrP^{Sc} accumulation (in red) in the ENS and CNS in natural scrapie-affected sheep of the PrP^{VRQ}/ PrP^{VRQ} genotype. Presence of PrP^{Sc} was determined by immunohistochemistry progression of accumulation studied by infection of several sheep and serially sacrificing sheep for study at* ***a*** *5 months;* ***b*** *14 months;* ***c*** *21 months and* ***d*** *26 months of age. Abbreviations: DMNV dorsal motor nucleus of the vagus; IMLC intermediolateral column; C, T and L cervical, thoracic and lumbal segments of the spinal cord; GN ganglion nodosum; R Rumen; Re Reticulum; Om Omasum; Ab Abomasum; Du Duodenum; Je Jejunum; il Ileum; Ca Caecum; Co Colon; Rec Rectum. Animal numbers are indicated on the left and on the right: the ENS and CNS of the same animal are aligned to the left or to the right. Only efferent nerve fibres are shown (reproduced from van Keulen, L.J., et al., Pathogenesis of natural scrapie in sheep. Arch Virol Suppl, 2000(16): p. 57-71, Wien-New York: Springer 2000 with permission).*

Prion Protein.

Prion is usually the term used to describe the agent (PrP^{Sc}) of TSEs or prion diseases. The term "prion protein" is used to denote the host-encoded PrP-molecule (PrP^{C}). The gene organisation and structure of this 'normal' cellular prion protein is strongly conserved among mammalian species. The sheep PrP-gene contains a promotor and three exons, with the sheep PrP open reading frame (PrP-ORF) encoding for a polypeptide of 256 amino acids (aa) entirely localised within exon III without interruption by intron sequences (Fig.4, [14]). The biosynthetic pathway followed by PrP^{C} can be compared to that of other membrane and secreted proteins [15, 16]; PrP^{C} is synthesised in the rough endoplasmatic reticulum, translocated to the Golgi and finally transported to the cell-surface. During biosynthesis several post-translational modifications take place during processing and maturation of the primary translational product (Fig.4). These posttranslational modifications include cleavage of N-terminal and C-terminal sequences, addition of asparagine (N) linked oligosaccharide chains (glycosylation) at two possible positions (aa184 and aa200), formation of a disulfide bridge (S-S) between the cysteines at aa182 and aa217 and addition of a glycolipid anchor (GPI-anchor) at the C-terminus. Both the core of the asparagine (N) linked oligosaccharide chains and the GPI-anchor are added in the ER and further modified in the Golgi. The GPI-anchor signal sequence is also thought to have the additional function of aiding in translocation of PrP^{C} to caveolae-like domains on the surface of the cell [17]. The mature cellular prion protein has a half-life of approximately 6 hours *in vivo* [18] or 60 minutes *in vitro* [16] and is expressed on the plasma membrane. Not all PrP^{C} molecules remain on the cell surface, some are internalised through endocytosis where PrP^{C} is either recycled back onto the plasma membrane or degraded. The morphological structures primarily responsible for PrP^{C} uptake appear to be clathrin coated pits and endocytic vesicles.

Most species have 5 octarepeats in the region of aa54-95 (Fig.4) near the N-terminus. In cattle 6 octarepeats are present but, the one extra octarepeat does not seem to have any effect on TSE susceptibility [19], whereas extra octarepeat insertions in humans (5 to 9 extra) have been associated with familial forms of Creutzfeldt-Jacob disease [20-24]. This is in concordance with the finding that insertions of four to six extra octarepeats in hamster PrP^{C} were necessary to see differences in location of PrP^{C}, spontaneous aggregation of PrP^{C} and increased protease-resistance of PrP^{C} expressed in tissue culture cells [25]. The octarepeats have been shown to bind copper ions (Cu2+), but the specific physiological relevance remains elusive.

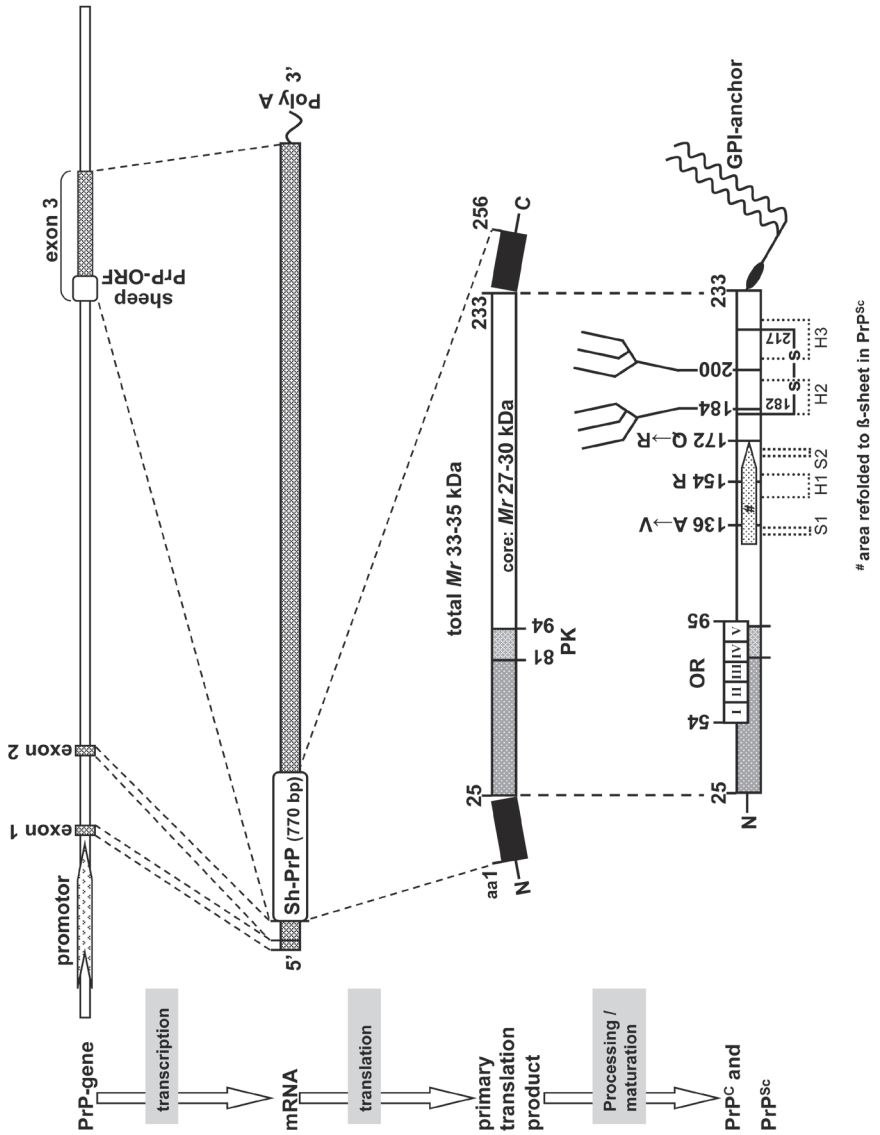


Figure 4. ***Schematic representation of structural organisation and maturation of PrP^C.*** *The PrP gene is transcribed to RNA and after splicing of the intron sequences the mRNA is translated by ribosomes in the endoplasmatic reticulum. The primary translation product is transported to the Golgi apparatus where further processing and posttranslational modification takes place (cleavage of signal sequences, glycosylation, disulphide-bridge formation and addition of GPI anchor). Mature PrP^C has a molecular weight (Mr) of 33-37 kDa and can be fully hydrolysed by proteinase-K (PK). PrP^{Sc} only differs in secondary and tertiary conformation, resulting in a partial PK resistance. PK cleaves about 6 kDa from the N-terminus of PrP^{Sc}, approximately between codons 81 and 94, dependant on conformation.*

Prion Protein Physiological Function and Interaction.

The exact function of the prion protein is (to date still) not fully known. However, the PrP gene is highly conserved and the structure and organisation suggests that its function is of importance. Even though the physiological function of PrP^C is elusive, PrP^C is well known for its capacity to bind copper ions (Cu^{2+}) at the octarepeats (Fig.4) containing four histidines in a general tandem motif P(G/H)GGGWGQ in mammals. The exact relation between PrP^C and copper is unclear, however there are clues that PrP^C is involved in Cu transport or sequestration [26] or vice versa that Cu^{2+} is involved in internalisation of PrP^C [27, 28]. Many studies link binding of Cu^{2+} to PrP^C with antioxidant activity either directly with PrP^C as the protective protein [29-31] or indirectly by PrP^C interaction with other proteins to elicit protection [32-39], implicating PrP^C as a protective protein during stress, in particular against copper toxicity. Furthermore, binding of Cu^{2+} to PrP^C seems to stabilize the prion protein in a more compact and thermodynamically stable intermediate, dependant on the genotype of the prion protein [40] and the N-terminal domain (containing the octarepeats) has an effect on aggregation kinetics and properties of the aggregates formed [41]. This suggests that scrapie resistance may be due to a change in stability of the 'resistant' prion protein aiding in metabolisation of the 'resistant' prion protein by the host before a proteinase resistant aggregate (PrP^{Sc}) is formed, explaining slower development of disease, with lower prion titers, in mice expressing N-terminally truncated prion protein [42].

Next to copper PrP^C has also been shown to interact with many different molecules. Because PrP^C is a GPI-anchored protein on the cell-surface and localised in rafts or caveolae it could be involved in signal transduction. PrP^C has been shown to interact (among others) with caveolin-1, the laminin receptor, synapsin 1b, Grb2 and STI1 (reviewed in [43, 44]).

It seems that PrP^C is involved in modulation of neuronal survival either by activating fyn tyrosine kinase (through interaction with caveolin-1) which is associated with cellular proliferation and cellular survival, by involvement in synapse formation and regulation of neurotransmitter release (synapsin Ib) or by mediating growth factor receptor signals (Gbr2). PrP^C also promotes neuritic outgrowth (interaction with the laminin receptor). Furthermore PrP^C is involved in neuroprotection through cAMP-dependant kinase (PKA) protection against anisomycin-mediated cell-death (apoptosis, by interacting with stress-inducible protein I [STI1]) or by preventing Bax mediated cell death ([45],

reviewed in [46]), but only when the PrP^C is matured and contains the octarepeat. These data suggest that PrP^C is involved in neuronal survival by sustaining synaptic function integrity.

These probable neuroprotective functions of PrP^C necessitate the need for new innovative therapeutic strategies that takes the (possible) importance of PrP^C functionality into account. Unfortunately, to date promising prion prophylactics found *in vitro* have failed to reap satisfactory results *in vivo*. Therefore gaining insight in which amino acids in PrP^C are capable of interacting and how these interaction domains are involved in prion protein biology and pathology are of importance. Understanding what affects PrP^C interaction may ultimately lead to therapeutic strategies halting PrP^{Sc} accumulation and even clearing hereof, whilst allowing the physiological function of PrP^C to remain intact or to be restored.

Biochemical and structural differences between PrP^C and PrP^{Sc}.

Even though PrP^C and PrP^{Sc} have the same genetic origin, distinct post-translational and biochemical differences are apparent between these two forms of the same protein (main differences summarised in table1, visualised in Fig.5). The normal cellular form of PrP (PrP^C) is by definition soluble and can be fully degraded by proteinase K. On the other hand, the disease associated prion protein (PrP^{Sc}) is insoluble even in mild detergents and forms precipitates or aggregates (including amyloid plaques or fibrils, reviewed in [47]). The high tendency to aggregate correlates with the (partial) proteinase K (PK) resistance of PrP^{Sc}. When PrP^{Sc} is digested with PK only 60-70 amino acids are cleaved from the N-terminus and occasionally a few amino acids from the C-terminus, which results in a core-protein of 27-30 kDa (Fig.4, [48]). However no covalent chemical differences have been detected between PrP^C and PrP^{Sc} to explain the observed differences in solubility and PK resistance [49, 50]. Differences do occur within the secondary, tertiary, and quaternary structure; PrP^C is a monomeric protein that consists of mainly α-helical structure (42%) and is considered the non-infectious form of PrP, whereas PrP^{Sc} has lower α-helix (20%) content and higher β-sheet (34%) content compared to PrP^C (reviewed in [47]) and is considered the infectious form of PrP (summarized in table 1).

Using recombinant hamster PrP, NMR spectroscopy could be applied to determine a three dimensional structure of hamster PrP^C, in particular the C-terminal part of hamster PrP (aa121-231, reviewed in [47]). Structure analysis of the 'full-length' mature prion protein revealed that the globular domain of PrP is contained within the C-terminal part (aa126-231) of PrP, whereas the N-terminus (aa23-125) is seemingly unstructured (Fig.5, left panel).

The structure consists of three α-helixes and two smaller β-sheets, which correlate with the predicted locations in sheep; β-sheet 1: aa132-134 (S1), α-helix 1: aa147-157(H1), β-sheet 2: aa164-166 (S2), α-helix 2: aa175-197 (H2) and α-helix 3: aa205-229 (H3). The two α-helixes H2 and H3 are connected by a disulfide bridge between the cysteines at aa182 (in H2) and aa217 (in H3).

Table 1. ***Biochemical and structural differences between PrP^C and PrP^{Sc}***

PrP^C	**PrP^{Sc}**
○ normal cellular form	○ disease associated form
○ non infectious	○ infectious
○ monomer	○ polymer (min. 5 for infectivity)
○ soluble	○ insoluble precipitates / aggregates amyloid plaques / fibrils
○ protease sensitive *Mr* (no PK) 33-35 kDa *Mr* (+PK) complete degredation	○ partially protease resistant *Mr* (no PK) 33-35 kDa *Mr* (+PK) 27-30 kDa
○ half life ~6 hours	○ half life » years
○ mainly a-helical structure ~ 42% α-helix ~ 3% ß-sheet	○ predominant ß-sheeted structure ~ 20% α-helix ~ 32% ß-sheet (ß-helix)

Interestingly, the region between S1 and H2 has some structural flexibility and this region has been implicated as the location of interest concerning the species barrier. Based on the NMR data for monomeric PrP^{C} concurrently with several experimental observations resulted in models for PrP^{Sc} (Fig.5, right panel). PrP^{Sc} contains less α-helical structure compared to PrP^{C}. Conservation of an antibody epitope in PrP^{C} and PrP^{Sc}, combined with immunochemical and structural characterization of binding of this antibody with both PrP^{C} and PrP^{Sc}, provided structural information PrP^{C} to PrP^{Sc} conversion; the two small β-sheets in PrP^{C} act as a starting point for unfolding/refolding after which the S1-H1-S2 part of the PrP^{C} structured domain is replaced by β-sheet structure ([51], Fig.4 and 5, right panel). However, the total structure of PrP^{Sc} and the nature of the β-structure were challenged by two-dimensional crystalline-like arrays of PrP^{Sc} -or PrP^{Sc}-like structures [52].

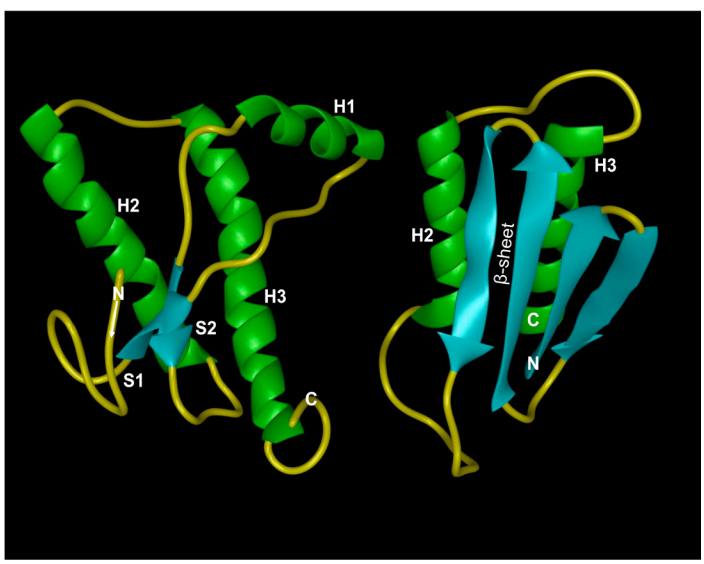


Figure 5. ***Three dimensional structures of PrP.*** *The left panel represents the structure as determined by NMR for recombinant hamster PrP^{C} [53]. Indicated are the positions (in order of sequence) of the N-terminus (N), β-sheet 1 (S1), α-helix 1 (H1), β-sheet 2 (S2), α-helix 2 (H2), α-helix 3 (H3) and the C-terminus. The right model represents the theoretical model of monomeric hamster PrP^{Sc} (Cohen et al. Cellular & Molecular Pharmacology, University of California, San Francisco USA). Indicated are the positions (in order of sequence) of the N-terminus (N), the newly formed β-sheet comprised of S1-H1-S2, α-helix 2 (H2), α-helix 3 (H3) and the C-terminus.*

Based on electron microscopic study of the crystalline-like arrays, an alternative model for PrP^{Sc} in which a different organisation of secondary and tertiary structures of the protein was proposed. This study argues that the β-sheet formation actually is a β-sheet-rich fold such as a parallel β-helix [52]. Further study of 119 all-β folds observed in globular proteins [54] revealed that if PrP^{Sc} follows a known protein fold it likely adopts a parallel left-handed β-helical fold (β-sandwich may also be possible) (Fig. 6). Parallel β-helices like these readily form trimers (Fig. 7), providing a natural template for a trimeric model of PrP^{Sc} that accommodates the PrP sequence from residues 89-175 in a β-helical conformation with the C-terminus retaining the disulfide-linked α-helical conformation observed in PrP^{C} [54]. This parallel β-helical model is consistent with many structural, biochemical, immunological, and propagation features of prions. Furthermore it takes in account the likely intermolecular stabilisation of the PrP^{Sc} structure and may even take in account the structural influence of the GPI-anchoring to the cell-membrane [47].

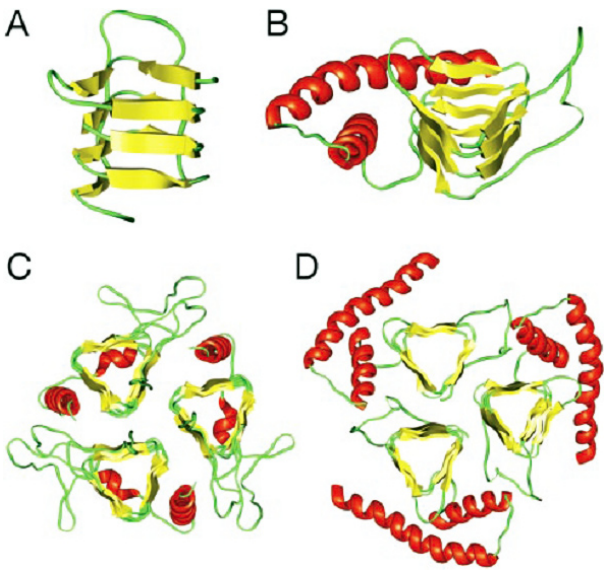


Figure 6. ***Modelling PrP residues 89–174 onto a left-handed β-helical fold.*** *(A) The β-helical model of the N-terminal part of PrP 27–30. (B) Model of the monomer of PrP 27–30. The β-helical region (residues 177–227) as determined by NMR spectroscopy (PDB ID code 1QM0) was linked to the β-helical model shown in A. (C) The crystal structure of the trimeric carbonic anhydrase from Methanosarcina thermophila (PDB ID code 1THJ). (D) Trimeric model of PrP 27–30 built by superimposing three monomeric models onto the coordinates of the Cα's of the 1THJ structure (figure by C. Govearts, reproduced from [54] with authors permission).*

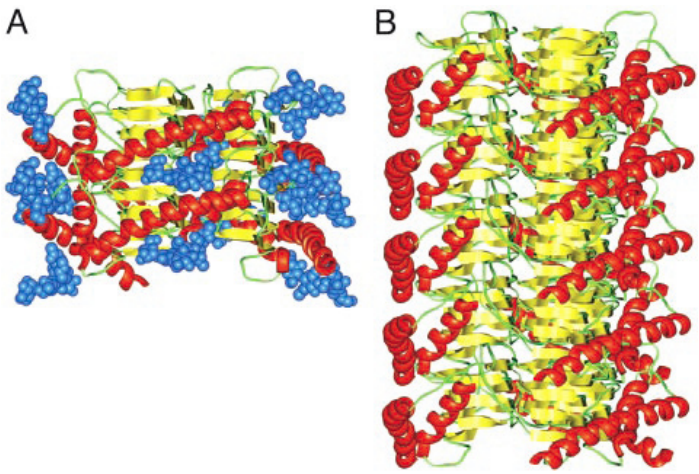


Figure 7. ***Fibrillization of the trimeric left-handed β-helical discs.*** *(A) Two discs of PrP 27–30 can assemble through polar backbone interactions between the lower β-helical rung of the top disk and the upper rung of the bottom disk. This assembly provides enough room for the α-helices to stack and the N-linked sugars to extend away from the center of the structure. (B) A model for the PrP 27–30 fiber was constructed by assembling five trimeric discs. For clarity, the sugars were omitted (figure by C. Govearts, reproduced from [54] with authors permission)*

Prion therapeutics.

When devising a therapeutic strategy against prion disease many obstacles need to be overcome. First of all the mechanism of prion propagation is still poorly understood. Even though knowledge on the infection-route and prion spread is increasing, several questions remain unanswered. However, it is now widely accepted that PrP^{Sc} is associated with prion disease. Therefore the protein-only theory stipulates that PrP^{Sc} is the only infectious particle in prion disease, resulting in therapeutic strategies that are aimed at PrP^{Sc} or preventing PrP^{Sc} accumulation.

Several characteristics need to be taken in account when devising a therapeutic strategy for prion disease. Diagnosis of TSE is first based on clinical features, which until the later stages of disease are rather general and applicable to several different (neurological) disorders. Only in the final stages of disease is clinical diagnosis specific, but the only definitive determination of prion disease to date is a post-mortem test detecting PrP^{Sc}.

Therefore, until the diagnosis can be made earlier in the disease pathogenesis, therapeutics must be able to cross the blood brain barrier (BBB) or otherwise be delivered to the central nervous system (CNS). Another aspect to take in account is the toxicity of the therapeutic; it needs to be effective at physiological concentrations and amounts after biological build-up (during administration) should not reach toxic levels. Furthermore, because the physiological function of PrP^{C} still remains elusive, an attempt to ensure PrP^{C} functionality or return of PrP^{C} functionality should be made. For example, when a therapeutic targets PrP^{C} and prevents either synthesis or trafficking to the cell surface, does expression and/or trafficking return to normal after treatment is finished? Or when the therapeutic interacts with PrP^{C}, PrP^{Sc} or interferes in the interaction between PrP^{C} and PrP^{Sc} does the therapeutic impair or abolish PrP^{C} functionality and can it be restored after treatment? Based on available pathogenic information, several therapeutic strategies can be proposed (reviewed in [55]): [i] prevention of infection at primary site of replication; [ii] preventing prion spread to the secondary lymphoid organs; [iii] prevention of follicular dendritic cell infection; [iv] preventing of spread though ENS by blocking the neuro-immune interface; [v] preventing prion spread through the peripheral nervous system; and [vi] preventing neuronal death. At a molecular level this could be realised by therapeutics that (reviewed in [55, 56]): [a] inhibit PrP^{C} - PrP^{Sc} interaction (i.e. by abrogation of PrP^{C} synthesis, by prevention of cellular trafficking to cell-surface of PrP^{C}, or by interfering in the interaction between PrP^{C}, PrP^{Sc} and other macromolecules involved in PrP conversion); [b] inhibition of PrP^{C} to PrP^{Sc} conversion (i.e. stabilisation or destabilisation of PrP^{C} preventing PrP^{C} conversion); [c] inducing clearance of PrP^{Sc} (i.e. sequestration of PrP^{Sc} or reverting PrP^{Sc} to a protease sensitive state); and [d] inhibit neurotoxic mediators synthesised in response to PrP^{Sc} accumulation. Taking in account the complexity of prion protein diseases, it may be very well possible that combining various strategies is necessary for therapy.

Prion protein conversion.

The 'protein-only' hypothesis [4] stipulates that the infectious particles (PrP^{Sc}) are able to convert host-encoded PrP^{C} into additional PrP^{Sc} particles equally capable of converting additional host-encoded PrP^{C} and thus self-replicating in the absence of nucleic acid. The mechanism involved in PrP^{C} to PrP^{Sc} remains elusive, however two mechanisms for prion propagation have been proposed. The main difference lies in the nature of the 'seed' In the first mechanism the infectious particle (PrP^{Sc}) comes in proximity of and forms a 'dimer' with PrP^{C}, imposes its structure, separates from the newly formed PrP^{Sc}

and becomes available to convert the next PrP^{C} molecule (Fig.7, panel 1). The second proposed mechanism is based on the principle that the 'seed' consists exclusively of a polymer from which nucleated polymerisation takes place, and the whole polymer acts as a seed for further conversion (Fig.7, panel 2). Some favour the dimerisation mechanism and consider aggregation and polymerisation an artefact of PrP^{Sc} isolation [57]. However, a multitude of experimental data indicates that nucleated polymerisation is the mechanism responsible for prion propagation [50, 58-63].

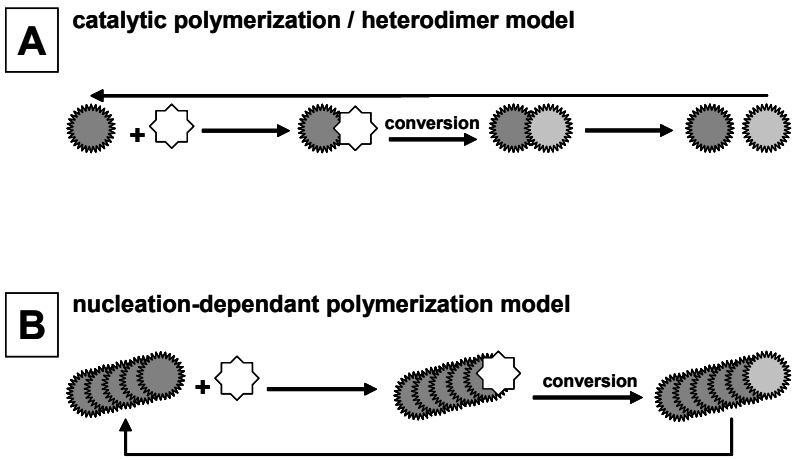


Figure 8. ***Schematic representation of two different conversion mechanisms.*** *Two different models for prion conversion have been proposed. The first model is the catalytic polymerization or heterodimer model [A] in which PrP^{C} (white) is bound by PrP^{Sc} (dark grey), resulting in conversion of PrP^{C}. This reaction results in a newly formed PrP^{Sc} (light grey), which separates from the original seed and both can recruit a new PrP^{C}. The second mechanism is the nucleation-dependant polymerization model [B], which correlates better with conversion data obtained in vitro. In this model a polymer of PrP^{Sc} recruits a PrP^{C} (at the polymerisation –or nucleation site) after which the tertiary structure of PrP^{C} is refolded to that of PrP^{Sc}. The now 'elongated' PrP^{Sc} polymer can recruit a new PrP^{C}.*

Recent *in vitro* conversion studies seem to strengthen the view that nucleated polymerisation is the mechanism responsible for prion propagation. A study on recombinant full-length PrP with an intact disulfide bond also shows that full-length PrP can adopt an amyloid structure reminiscent of PrP^{Sc} and also showed that cleavage of the N-terminal region renders amyloid fibres susceptible to breakage of these fibres and may be responsible in creating new seeds for prion propagation [64]. Another

study using the protein misfolding cyclic amplification reaction (PMCA) developed by Saborio *et al*. [65] showed that autocatalytic propagation of misfolded prion protein during continuous dilution could only endure when the reactions were sonicated [66], implicating that formation of the necessary extra seeds only occurs when aggregates are broken. Furthermore, study of human 145stop variant (mutation responsible for Gerstmann-Straussler-Scheinker-like disease) resulted in spontaneous conversion to a fibrillar form, characterized by a protein-dependant lag phase and had characteristics of nucleation-dependant polymerisation [63]. These data taken together strongly suggests that prion propagation takes place through nucleation-dependant polymerisation.

Influence of polymorphisms in PrP (PRNP) on TSE susceptibility. The human gene, *PRNP*, is located on the short arm of chromosome 20. In Creutzfeldt-Jacob disease cases in general, there is an over representation of homozygousity at amino acid position (aa)129 either for methionine or valine. However, polymorphism at aa129 is not solely responsible, but undoubtedly important in disease susceptibility and pathogenesis. In sporadic CJD 83% of the studied cases were homozygous for methionine and 9% of these cases were homozygous for valine at aa129. The relative risk factor for M/M vs. M/V is 11:1 and V/V vs. M/V is 4:1 showing homozygous methionine at 129 is an increased risk factor, but that homozygousity in general is a risk-factor in sporadic CJD [67]. Hereditary (familial) Creutzfeldt-Jacob disease is associated with over 20 PRNP haplotypes (reviewed in [5, 68]). These mutations consist of point mutations involving single amino acid changes or deletion and insertion mutations involving the reduction or elongation of the number of octarepeats. These mutations combined with aa129 polymorphism and the type of PrP^{Sc} determines the disease phenotype (severity in neuronal loss and astrogliosis). Genetic analysis of patients diagnosed with variant CJD, which is thought to be a result of ingestion of BSE infected beef or beef products, are homozygous for methionine at aa129 [68]. However, studies of iatrogenic CJD (in growth hormone recipients) and kuru has revealed that homozygous methionine at aa129 shortens the incubation period, but that later cases also involved homozygous valine at aa129 and that heterozygous methionine/valine at aa129 resulted in the longest incubation period [68]. Furthermore, in iatrogenic CJD homozygosity for valine at aa129 is more frequently found compared to sCJD [5]. Therefore, it cannot be ruled out that cases of vCJD will be found with longer incubation periods in persons homozygous for valine or heterozygous methionine/valine at aa129.

To date, genetic analysis of the bovine *PRNP* gene has not resulted in determining polymorphisms linked to susceptibility to BSE. However, one study genetically compared the whole gene including the exons and the promotor region of *PRNP*. Amongst other

polymorphisms, a 23bp insertion/deletion in the promotor region was found, which correlated significantly with BSE susceptibility [69]. This 23bp insertion/deletion was more frequently found in healthy cattle and in the region of this mutation the bovine *PRNP* contains a retroviral sequence. It has been shown that such retroviral sequences are an essential part of the promotor and as such the 23bp insertion/deletion may be a regularitory mutation. Further research into the 23bp insertion/deletion, which contains binding sites for the transcription factors PR58 and SP1, shows that this mutation modulates the expression levels of *PRNP in vitro* [70]. The *in vivo* data suggests that this genotype may contribute to the high variance of *PRNP* expression observed in intestinal lymph nodes.

Scrapie in small ruminants (e.g. sheep) is one of the best documented models for natural TSE transmission. Polymorphisms in sheep PrP have been shown to be of importance in both interspecies and intraspecies transmissibilities [71]. Polymorphisms in the gene coding for the prion protein (*PNRP*) dictate susceptibility of sheep to scrapie and over 20 different, naturally occurring polymorphisms (only one mutation per allele) of PrP have been described [72-80]. The modulating effects on the relative susceptibility of sheep to scrapie of these naturally occurring polymorphisms in sheep PrP has been studied in either epidemiological studies of natural scrapie outbreaks, in experimental transmissions to and from sheep, and in cell-free conversion assays [72, 81-85]. These studies have established that polymorphisms at sheep PrP amino acid position 136, 154 and 171 (Fig.4) are most relevant in differential TSE susceptibility and that an alanine at position 136, arginine at position 154 and glutamine at position 171 (ARQ) is the phylogenetic wild type of PrP (wt), with intermediate susceptibility to scrapie. The polymorphism associated with increased susceptibility to scrapie is the substitution alanine with valine at codon 136 (VRQ; 136V) and thus far the only polymorphism shown to be associated with decreased susceptibility or even resistance to natural scrapie is the substitution of glutamine with arginine at codon 171 (ARR; 171R). However, the exact molecular mechanism behind disease associated polymorphism modulation of prion protein conversion remains unknown [86-88].

Influence of glycosylation on prion propagation. N-linked glycosylation of PrP has a still elusive function, but the glycosylation sites are (like the protein) conserved in all mammalian PrP genes and thus seem of importance in prion protein biology. Furthermore, the rate of glycosylation is variable but reproducible, resulting in distinct subsets of non-glycosylated, mono-glycosylated and di-glycosylated prion protein. Differences in glycan composition between PrP^{C} and PrP^{Sc} have been observed, but the cause of this shift in composition remains unknown. Glycosylation differences have also

been observed in the GPI anchor, of which the encoding residues in turn have an effect on N-linked glycosylation, suggesting that glycosylation of the sites occurs cotranslationally, prior to the addition of the GPI moiety (reviewed in [89]). Although glycosylation is not required for prion propagation, several studies have shown that differences in glycosylation can affect prion propagation indirectly, likely due to different localization of PrP^{C} as a result of altered post-translational modifications in general (reviewed in [89]). In absence of an agent-specific coding molecule containing nucleic acids, it is clear that PrP^{Sc} conformation and glycosylation, together with host-specific factors including PrP primary structure and host-specific glycosylation, contribute to the molecular basis of TSE strain diversity (reviewed in [89]). Furthermore, *in vitro* conversion assays have shown that even though amino acid sequence compatibility remains the primary determinant of PrP^{C} to PrP^{Sc} conversion, the glycosylation state of PrP can influence the initial binding event and affinity of this interaction as well as influence the conformation of protinase K resistant PrP (PrP^{res}) generated in the cell-free conversion assay (reviewed in [89]). All the data described indicate a functional role for PrP glycosylation in the maintenance of prion strains and whether, and if so, how efficient the infectious agent can cross the species barrier.

Scope of the thesis.

It is abundantly clear that the exact molecular processes behind PrP^{C} into PrP^{Sc} conversion are not clearly understood. Correlations between prion protein polymorphisms and disease have been found, however in what way these polymorphisms influence the conversion processes remains an enigma; is stabilization or destabilization of the prion protein the basis for a higher conversion propensity?

Apart from the disease associated polymorphisms of the prion protein, the molecular processes underlying conversion are not understood. There are some notions as to which regions of the prion protein are involved in refolding of PrP^{C} into PrP^{Sc} and were the most drastic structural changes take place. Direct interactions between PrP^{C} molecules and/or PrP^{Sc} are likely at the basis of conversion, however which specific amino acid domains are involved and to what extent these domains contribute to conversion resistance/sensitivity of the prion protein or the species barrier is still unknown. Furthermore the physiological function of PrP^{C} is also still enigmatic; does refolding of the protein play a role in its function? How does interfering with PrP^{C} function by therapeutic treatment affect the treated individual? Until such questions are answered it seems prudent to design

therapeutic therapies that circumvent total ablation of PrP^C or at least focus should be on strategies that allow for retention or reacquiring of PrP^C physiological function.

Several basic questions remain to be answered and this thesis aimed to study the processes involved in PrP^C to PrP^{Sc} conversion. First the influence of disease associated polymorphisms on binding and/or conversion was studied. Based on these results this thesis further aimed to determine which specific amino acid sequences (domains) are involved in PrP interaction and how these specific interaction domains influence conversion of PrP^C into PrP^{Sc}. Answering these questions will allow for a more detailed understanding of the conversion processes, which in turn should allow for improvement of currently available therapeutics, as well as provide clues for designing novel therapeutic compounds or strategies.

These studies culminated into the following publications/chapters;

Chapter 2: *How can TSE propagation/conversion best be studied?* This chapter is a detailed review of *in vitro* systems used to study TSEs replication and the process involved in prion protein conversion. Here also the advantages and disadvantages of these *in vitro* systems are discussed, and for several applications species specificity, polymorphism specificity, strain specificity, and the potential of these systems in screening and discovery of TSE therapeutics is discussed.

Chapter 3: *Which particular part of the conversion process is affected by scrapy-susceptibilty linked polymorphisms?* This chapter describes the study trying to elucidate the effects of disease associated polymorphisms on conversion, specifically focusing on the molecular interactions between PrP^C and PrP^{Sc}. Determining whether differential binding efficiencies of sheep PrP^C to PrP^{Sc} are influenced by scrapie-susceptibility linked polymorphisms (encoded at amino acid positions 136, 154 and 171) and can explain the observed differential conversion efficiencies of sheep PrP.

Chapter 4: *Which specific amino acid domains are involved in prion protein interaction?* This chapter describes the study to systematically map all possible amino acids (aa) that are involved in PrP interaction (which aa of PrP are capable of interaction) using ovine -and bovine solid-phase PrP peptide-arrays consisting of 15-mer overlapping peptides. The exact mechanisms behind the conversion processes are far from understood. However it seems likely that these processes are determined by similarities as well as

strain dependent variations in the PrP structure. Mapping aa involved in PrP interaction should provide insights in which similarities or differences may be of importance in the conversion processes.

Chapter 5: Interactions between proteins are of importance for most if not all biological processes in living cells. Consequently, study of these interactions and elucidating the contributing amino acid sequences involved may lead to improved understanding of the processes underlying disease and may provide the basis for new therapeutic approaches. The novel use of peptide-arrays as described in chapter 4 may be applied to other proteins. Therefore this chapter entails a more methodological description of our novel application of peptide-arrays, focussing on practical points of importance.

Chapter 6: *How do specific interaction domains affect conversion, and at which stage of conversion are the interaction domains of importance?* This chapter describes the study to elucidate how the previously determined interaction domains (chapter 4) affect two processes; (self-) interaction and conversion. These two processes of importance in prion disease development were studied using peptides from selected regions of PrP likely involved in the conversion processes and based on the interaction domains and properties reported in literature, six ovine PrP regions were selected for testing.

Chapter 7: *How may the studies described in this thesis be of use in improving current and development of novel therapeutic strategies?* The current "state of the art" of prion disease therapeutic strategies and compounds is reviewed in detail in this chapter. The most promising therapeutics are described listing both pros and cons of each therapeutic as well as limitations of use and necessary studies needed to fully assess feasibility of use. Furthermore, the results of the studies presented in this thesis are discussed in relation to further development of current and new therapeutic strategies or compounds.

References.

1. Alper, T., D.A. Haig, and M.C. Clarke, *The exceptionally small size of the scrapie agent.* Biochem Biophys Res Commun, 1966. 22(3): p. 278-84.
2. Alper, T., et al., *Does the agent of scrapie replicate without nucleic acid?* Nature, 1967. 214(90): p. 764-6.
3. Griffith, J.S., *Self-replication and scrapie.* Nature, 1967. 215(105): p. 1043-4.
4. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. 216(4542): p. 136-44.
5. Takemura, K., et al., *An overview of transmissible spongiform encephalopathies.* Anim Health Res Rev, 2004. 5(2): p. 103-24.
6. Pedersen, N.S. and E. Smith, *Prion diseases: epidemiology in man.* Apmis, 2002. 110(1): p. 14-22.
7. Will, R.G., et al., *A new variant of Creutzfeldt-Jakob disease in the UK.* Lancet, 1996. 347(9006): p. 921-5.
8. Lasmezas, C.I., et al., *BSE transmission to macaques.* Nature, 1996. 381(6585): p. 743-4.
9. Ironside, J.W., *Neuropathological findings in new variant CJD and experimental transmission of BSE.* FEMS Immunol Med Microbiol, 1998. 21(2): p. 91-5.
10. Cashman, N.R. and B. Caughey, *Prion diseases--close to effective therapy?* Nat Rev Drug Discov, 2004. 3(10): p. 874-84.
11. van Keulen, L.J., et al., *Pathogenesis of natural scrapie in sheep.* Arch Virol Suppl, 2000(16): p. 57-71.
12. van Keulen, L.J., M.E. Vromans, and F.G. van Zijderveld, *Early and late pathogenesis of natural scrapie infection in sheep.* Apmis, 2002. 110(1): p. 23-32.
13. Bruce, M.E., et al., *Follicular dendritic cells in TSE pathogenesis.* Immunol Today, 2000. 21(9): p. 442-6.
14. Goldmann, W., *PrP gene and its association with spongiform encephalopathies.* Br Med Bull, 1993. 49(4): p. 839-59.
15. Harris, D.A., *Cellular biology of prion diseases.* Clin Microbiol Rev, 1999. 12(3): p. 429-44.
16. Harris, D.A., *Trafficking, turnover and membrane topology of PrP.* Br Med Bull, 2003. 66: p. 71-85.
17. Kaneko, K., et al., *COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform.* Proc Natl Acad Sci U S A, 1997. 94(6): p. 2333-8.
18. Bennett, A.D., C.R. Birkett, and C.J. Bostock, *Molecular biology of scrapie-like agents.* Rev Sci Tech, 1992. 11(2): p. 569-603.

19. Neibergs, H.L., et al., *Polymorphism analysis of the prion gene in BSE-affected and unaffected cattle.* Anim Genet, 1994. 25(5): p. 313-7.
20. Campbell, T.A., et al., *A prion disease with a novel 96-base pair insertional mutation in the prion protein gene.* Neurology, 1996. 46(3): p. 761-6.
21. Cochran, E.J., et al., *Familial Creutzfeldt-Jakob disease with a five-repeat octapeptide insert mutation.* Neurology, 1996. 47(3): p. 727-33.
22. Goldfarb, L.G., et al., *Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene.* Proc Natl Acad Sci U S A, 1991. 88(23): p. 10926-30.
23. Goldfarb, L.G., et al., *A new (two-repeat) octapeptide coding insert mutation in Creutzfeldt-Jakob disease.* Neurology, 1993. 43(11): p. 2392-4.
24. Laplanche, J.L., et al., *Two novel insertions in the prion protein gene in patients with late-onset dementia.* Hum Mol Genet, 1995. 4(6): p. 1109-11.
25. Priola, S.A. and B. Chesebro, *Abnormal properties of prion protein with insertional mutations in different cell types.* J Biol Chem, 1998. 273(19): p. 11980-5.
26. Brown, D.R., *Prion protein expression aids cellular uptake and veratridine-induced release of copper.* J Neurosci Res, 1999. 58(5): p. 717-25.
27. Perera, W.S. and N.M. Hooper, *Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region.* Curr Biol, 2001. 11(7): p. 519-23.
28. Pauly, P.C. and D.A. Harris, *Copper stimulates endocytosis of the prion protein.* J Biol Chem, 1998. 273(50): p. 33107-10.
29. Brown, D.R., et al., *Normal prion protein has an activity like that of superoxide dismutase.* Biochem J, 1999. 344 Pt 1: p. 1-5.
30. Brown, D.R., C. Clive, and S.J. Haswell, *Antioxidant activity related to copper binding of native prion protein.* J Neurochem, 2001. 76(1): p. 69-76.
31. Brown, D.R., R.S. Nicholas, and L. Canevari, *Lack of prion protein expression results in a neuronal phenotype sensitive to stress.* J Neurosci Res, 2002. 67(2): p. 211-24.
32. Zeng, F., et al., *Tethering the N-terminus of the prion protein compromises the cellular response to oxidative stress.* J Neurochem, 2003. 84(3): p. 480-90.
33. Wong, B.S., et al., *Differential contribution of superoxide dismutase activity by prion protein in vivo.* Biochem Biophys Res Commun, 2000. 273(1): p. 136-9.
34. Walz, R., et al., *Increased sensitivity to seizures in mice lacking cellular prion protein.* Epilepsia, 1999. 40(12): p. 1679-82.
35. Sakudo, A., et al., *Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line.* Biochem Biophys Res Commun, 2003. 308(3): p. 660-7.

36. Rachidi, W., et al., *Expression of prion protein increases cellular copper binding and antioxidant enzyme activities but not copper delivery.* J Biol Chem, 2003. 278(11): p. 9064-72.
37. Miele, G., et al., *Ablation of cellular prion protein expression affects mitochondrial numbers and morphology.* Biochem Biophys Res Commun, 2002. 291(2): p. 372-7.
38. Klamt, F., et al., *Imbalance of antioxidant defense in mice lacking cellular prion protein.* Free Radic Biol Med, 2001. 30(10): p. 1137-44.
39. Dupuis, L., et al., *Loss of prion protein in a transgenic model of amyotrophic lateral sclerosis.* Mol Cell Neurosci, 2002. 19(2): p. 216-24.
40. Wong, E., A.M. Thackray, and R. Bujdoso, *Copper induces increased beta-sheet content in the scrapie-susceptible ovine prion protein PrPVRQ compared with the resistant allelic variant PrPARR.* Biochem J, 2004. 380(Pt 1): p. 273-82.
41. Frankenfield, K.N., E.T. Powers, and J.W. Kelly, *Influence of the N-terminal domain on the aggregation properties of the prion protein.* Protein Sci, 2005. 14(8): p. 2154-66.
42. Flechsig, E., et al., *Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice.* Neuron, 2000. 27(2): p. 399-408.
43. Brown, D.R., *Role of the prion protein in copper turnover in astrocytes.* Neurobiol Dis, 2004. 15(3): p. 534-43.
44. Lee, K.S., et al., *Towards cellular receptors for prions.* Rev Med Virol, 2003. 13(6): p. 399-408.
45. Bounhar, Y., et al., *Prion protein protects human neurons against Bax-mediated apoptosis.* J Biol Chem, 2001. 276(42): p. 39145-9.
46. Roucou, X., M. Gains, and A.C. LeBlanc, *Neuroprotective functions of prion protein.* J Neurosci Res, 2004. 75(2): p. 153-61.
47. Riesner, D., *Biochemistry and structure of PrP(C) and PrP(Sc).* Br Med Bull, 2003. 66: p. 21-33.
48. Parchi, P., et al., *Different patterns of truncated prion protein fragments correlate with distinct phenotypes in P102L Gerstmann-Straussler-Scheinker disease.* Proc Natl Acad Sci U S A, 1998. 95(14): p. 8322-7.
49. Stahl, N., et al., *Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing.* Biochemistry, 1993. 32(8): p. 1991-2002.
50. Caughey, B., et al., *Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state.* Chem Biol, 1995. 2(12): p. 807-17.
51. Eghiaian, F., et al., *Insight into the PrPC-->PrPSc conversion from the structures of antibody-bound ovine prion scrapie-susceptibility variants.* Proc Natl Acad Sci U S A, 2004. 101(28): p. 10254-9.

52. Wille, H., et al., *Structural studies of the scrapie prion protein by electron crystallography.* Proc Natl Acad Sci U S A, 2002. 99(6): p. 3563-8.

53. James, T.L., et al., *Solution structure of a 142-residue recombinant prion protein corresponding to the infectious fragment of the scrapie isoform.* Proc Natl Acad Sci U S A, 1997. 94(19): p. 10086-91.

54. Govaerts, C., et al., *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A, 2004. 101(22): p. 8342-7.

55. Dormont, D., *Approaches to prophylaxis and therapy.* Br Med Bull, 2003. 66: p. 281-92.

56. Rossi, G., et al., *Therapeutic approaches to prion diseases.* Clin Lab Med, 2003. 23(1): p. 187-208.

57. Kaneko, K., et al., *Prion protein (PrP) synthetic peptides induce cellular PrP to acquire properties of the scrapie isoform.* Proc Natl Acad Sci U S A, 1995. 92(24): p. 11160-4.

58. Lansbury, P.T., Jr. and B. Caughey, *The chemistry of scrapie infection: implications of the 'ice 9' metaphor.* Chem Biol, 1995. 2(1): p. 1-5.

59. Caughey, B., et al., *Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies.* J Virol, 1997. 71(5): p. 4107-10.

60. Come, J.H., P.E. Fraser, and P.T. Lansbury, Jr., *A kinetic model for amyloid formation in the prion diseases: importance of seeding.* Proc Natl Acad Sci U S A, 1993. 90(13): p. 5959-63.

61. Gajdusek, D.C., *Transmissible and non-transmissible amyloidoses: autocatalytic post-translational conversion of host precursor proteins to beta-pleated sheet configurations.* J Neuroimmunol, 1988. 20(2-3): p. 95-110.

62. Kocisko, D.A., P.T. Lansbury, Jr., and B. Caughey, *Partial unfolding and refolding of scrapie-associated prion protein: evidence for a critical 16-kDa C-terminal domain.* Biochemistry, 1996. 35(41): p. 13434-42.

63. Kundu, B., et al., *Nucleation-dependent conformational conversion of the Y145Stop variant of human prion protein: structural clues for prion propagation.* Proc Natl Acad Sci U S A, 2003. 100(21): p. 12069-74.

64. Bocharova, O.V., et al., *Synthetic prions generated in vitro are similar to a newly identified subpopulation of PrPSc from sporadic Creutzfeldt-Jakob Disease.* Protein Sci, 2005. 14(5): p. 1222-32.

65. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. 411(6839): p. 810-3.

66. Piening, N., et al., *Breakage of PrP aggregates is essential for efficient autocatalytic propagation of misfolded prion protein.* Biochem Biophys Res Commun, 2005. 326(2): p. 339-43.

67. Windl, O., et al., *Genetic basis of Creutzfeldt-Jakob disease in the United Kingdom: a systematic analysis of predisposing mutations and allelic variation in the PRNP gene.* Hum Genet, 1996. 98(3): p. 259-64.
68. Gambetti, P., P. Parchi, and S.G. Chen, *Hereditary Creutzfeldt-Jakob disease and fatal familial insomnia.* Clin Lab Med, 2003. 23(1): p. 43-64.
69. Sander, P., et al., *Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds.* Neurogenetics, 2004. 5(1): p. 19-25.
70. Sander, P., et al., *Bovine prion protein gene (PRNP) promoter polymorphisms modulate PRNP expression and may be responsible for differences in BSE susceptibility.* J Biol Chem, 2005.
71. Bossers, A., et al., *In vitro conversion of normal prion protein into pathologic isoforms*, in *Clin Lab Med*, B. Ghetti, MD and P. Piccardo, MD, Editors. 2003, W.B. Saunders Company, a division of Elsivier Science. p. 227-47.
72. Bossers, A., et al., *PrP genotype contributes to determining survival times of sheep with natural scrapie.* J Gen Virol, 1996. 77 (Pt 10): p. 2669-73.
73. Belt, P.B.G.M., et al., *PrP allelic variants associated with natural scrapie.*, in *Bovine Spongiform Encephalopathy; The BSE dilemma*, C.J. Gibbs Jr., Editor. 1996, Springer: New York. p. 294-305.
74. Goldmann, W., et al., *Two alleles of a neural protein gene linked to scrapie in sheep.* Proc Natl Acad Sci U S A, 1990. 87(7): p. 2476-80.
75. Goldmann, W., et al., *Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene.* J Gen Virol, 1991. 72 (Pt 10): p. 2411-7.
76. Junghans, F., et al., *Genotyping of German sheep with respect to scrapie susceptibility.* Vet Rec, 1998. 143(12): p. 340-1.
77. Elsen, J.M., et al., *Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov.* Arch Virol, 1999. 144(3): p. 431-45.
78. Thorgeirsdottir, S., et al., *PrP gene polymorphism and natural scrapie in Icelandic sheep.* J Gen Virol, 1999. 80 (Pt 9): p. 2527-34.
79. Tranulis, M.A., et al., *Prion protein gene polymorphisms in sheep with natural scrapie and healthy controls in Norway.* J Gen Virol, 1999. 80 (Pt 4): p. 1073-7.
80. O'Rourke, K.I., et al., *Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue.* J Clin Microbiol, 2000. 38(9): p. 3254-9.
81. Goldmann, W., et al., *PrP genotypes and the Sip gene in Cheviot sheep form the basis for scrapie strain typing in sheep.* Ann N Y Acad Sci, 1994. 724: p. 296-9.
82. Hunter, N., et al., *Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes.* Arch Virol, 1996. 141(5): p. 809-24.

83. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.

84. Bossers, A., *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie*. 1999, University of Utrecht.

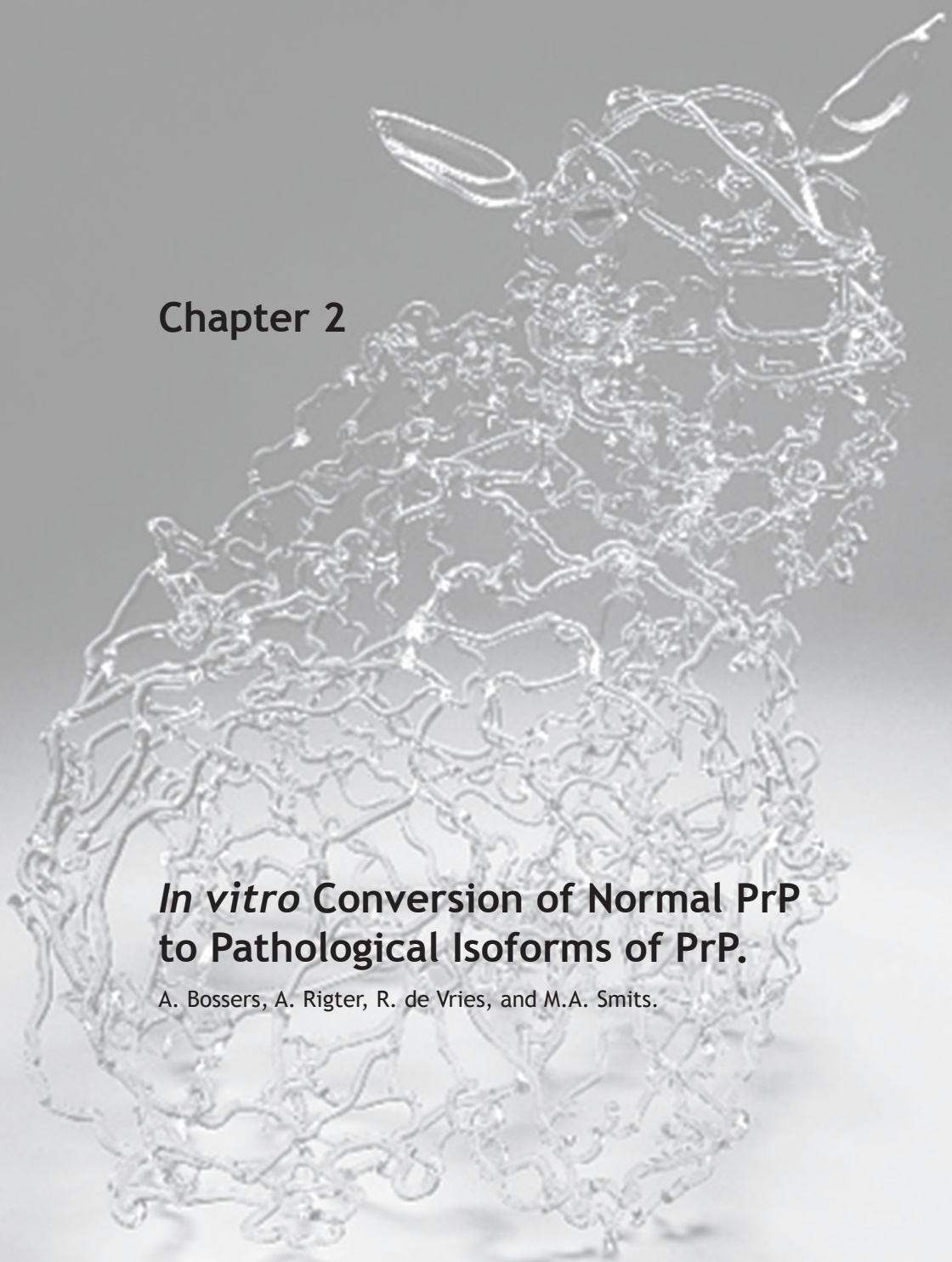
85. Bossers, A., R. de Vries, and M.A. Smits, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol, 2000. 74(3): p. 1407-14.

86. Dubois, M.A., et al., *Multiplicative genetic effects in scrapie disease susceptibility.* C R Biol, 2002. 325(5): p. 565-70.

87. Tranulis, M.A., *Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep.* Apmis, 2002. 110(1): p. 33-43.

88. Sabuncu, E., et al., *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol, 2003. 77(4): p. 2696-700.

89. Lawson, V.A., et al., *Prion protein glycosylation.* J Neurochem, 2005. 93(4): p. 793-801.



Chapter 2

In vitro Conversion of Normal PrP to Pathological Isoforms of PrP.

A. Bossers, A. Rigter, R. de Vries, and M.A. Smits.

Clinics in Laboratory Medicine. 2003 Mar; 23(1):227-47. Review.

In vitro Conversion of Normal PrP to Pathological Isoforms of PrP.

A. Bossers, A. Rigter, R. de Vries, and M.A. Smits.

Abstract.

The in vitro conversion techniques in cell-free and cell culture systems have provided tools to adequately study the underlying mechanism of TSEs, namely PrP conversion. These systems also have provided tools that make it easier to study the interspecies and intraspecies transmissibilities of TSEs. Finally, these systems also may assist in the discovery of TSE therapeutic strategies and in the development of extremely sensitive TSE detection techniques. In vivo TSE transmission studies are limited to (transgenic) animals (mostly mice). Although the cell culture systems also are restricted in their species-range (mostly mouse), the currently used cell-free systems allow studying of almost all possible species barriers (including the potential transmission of various TSEs to humans). One advantage of the cell culture systems, however, is that they generate do novo TSE infectivity. Studies using cell cultures also take into account several cofactors in addition to PrP that might be involved in replication the TSE agent. Although the in vitro systems provide accurate tools to study TSE agent parameters, they mainly or only focus on the molecular processes of PrP conversion. Other factors (i.e., host genetic factors) that, for example, determine the differential uptake of the TSE agent from the environment, might play an additional role in determining the susceptibility of hosts for TSEs and on the transmission of the disease among individuals.

Introduction.

Transmissible Spongiform Encephalopathies (TSEs) or prion diseases are fatal neurodegenerative diseases in animals and humans that can be transmitted by natural and experimental infections. Prion diseases are characterized by the accumulation of an abnormal isoform of the host-encoded cellular prion protein (PrP^{C}) mainly in, but not limited to, tissues of the central nervous system. The exact nature of the causative agent remains unknown, but most evidence supports a proteinaceous nature [1]. The pathologically folded isoform of PrP (PrP^{Sc}) seems to form the major, if not the only, component of the agent transmitting the disease [2].

Although much research has been done on the susceptibilities of hosts for TSEs and the transmissibilities of TSEs *in vivo*, only a few *in vitro* systems have been described suitable to study the biological characteristics of TSEs. The *in vitro* systems have several advantages over *in vivo* systems. They are generally rapid and suitable systems to measure various TSE associated parameters at a medium to high throughput level. They are very specific and various parameters can easily be altered, and because these systems do not require laboratory animals they are animal friendly. These *in vitro* systems fit essentially into two categories: cell culture systems and cell-free systems. Although in classical virology the cell culture systems are the favorite techniques for studying and assaying the infectious agent, TSE tissue culture systems are much more difficult to set-up, to maintain and/or to trigger to produce TSE infectivity. Most of the cell culture systems were initially developed for mouse scrapie and only very recently a cell culture system has been reported that replicates sheep scrapie after passage through transgenic mice overexpressing sheep PrP [3]. Especially the persistently infected mouse neuroblastoma cell culture systems have proven useful to assess various parameters in TSE agent replication (see Priola *et al.* 1999 for a review).

Another category of the *in vitro* systems are the so-called cell-free systems in which purified protein components or cell lysates are used to monitor PrP conversion [4-6]. The most frequently used system is based on monitoring the formation of protease resistant radio labeled PrP after mixing radio labeled PrP^{C} with purified non-radio labeled PrP^{Sc} [4]. Compared to cell culture systems, with this cell-free system a much wider range of species can be studied since it allows studying TSE transmissibility and susceptibility parameters at the molecular level of virtually any host-species and agent strains. Although the cell-free system was initially developed for hamster scrapie [4] it quickly turned out to be a system that demonstrated a number of biologically relevant parameters for TSEs including species-specificity and species-barriers, the specificity at the level of single

polymorphisms, and even TSE strain-specificity [7-11]. The system has been used to assess various species-barriers for TSEs like bovine spongiform encephalopathy (BSE or 'mad-cow disease'), chronic wasting disease (CWD), and sheep scrapie to many different 'hosts' like cattle, deer, sheep, mice, and humans [11, 12].

PrP conversion.

PrP^{Sc} is derived from PrP^{C} by a post-translational process involving conformational changes only. According to the (protein-only) prion hypothesis, infectious prion particles consist of PrP^{Sc} molecules that are able to convert the harmless host PrP^{C} into additional copies of PrP^{Sc}, thus replicating itself in the absence of nucleic acid (Fig. 1 panel 1). The interaction between PrP^{C} and PrP^{Sc} and the subsequent conversion are in general most efficient when the two isoforms have the same primary amino acid sequence [13], giving a molecular basis to the species barrier.

In inherited prion diseases, the mutated host PrP^{C} is thought to convert spontaneously into the pathogenic PrP^{Sc}, thereby generating a "seed" for further conversion of PrP^{C} into PrP^{Sc} (Fig. 1 panel 2). Alternative mechanisms underlying the 'genetic' forms have also been proposed in which polymorphisms (mutations that lead to amino acid alterations) in PrP alter the PrP^{C} membrane topology (^{Ctm}PrP) which would represent a key intermediate in the pathway of prion-induced neurodegeneration even in the absence of detectable PrP^{Sc} [14]. The aberrant formation of altered transmembrane forms seems to be restricted however to polymorphisms located in or near the conserved membrane-spanning element of PrP [15]. PrP mutations -especially those associated with familial forms of TSEs- can lead to aberrant behavior of PrP^{C} that results in the spontaneous formation of PrP^{Sc}-like properties as detergent insolubility, increased protease-resistance, and tight association of PrP to the cell membrane even after cleavage of its glycosylphosphatidylinositol anchor [16] (see also another chapter of this issue).

Polymorphisms in PrP may not only result in genetic forms of TSE. Some mutations in PrP may make PrP^{C} more prone to convert into PrP^{Sc} after interaction with the TSE agent, while other mutations make PrP^{C} less prone to convert into PrP^{Sc} (Fig. 1 panel 3). Such mutations cause within-species variability in the susceptibility for prion diseases and the subsequent transmission. Thus, polymorphisms may lead to altered incubation periods and/or incomplete transmissions of the disease.

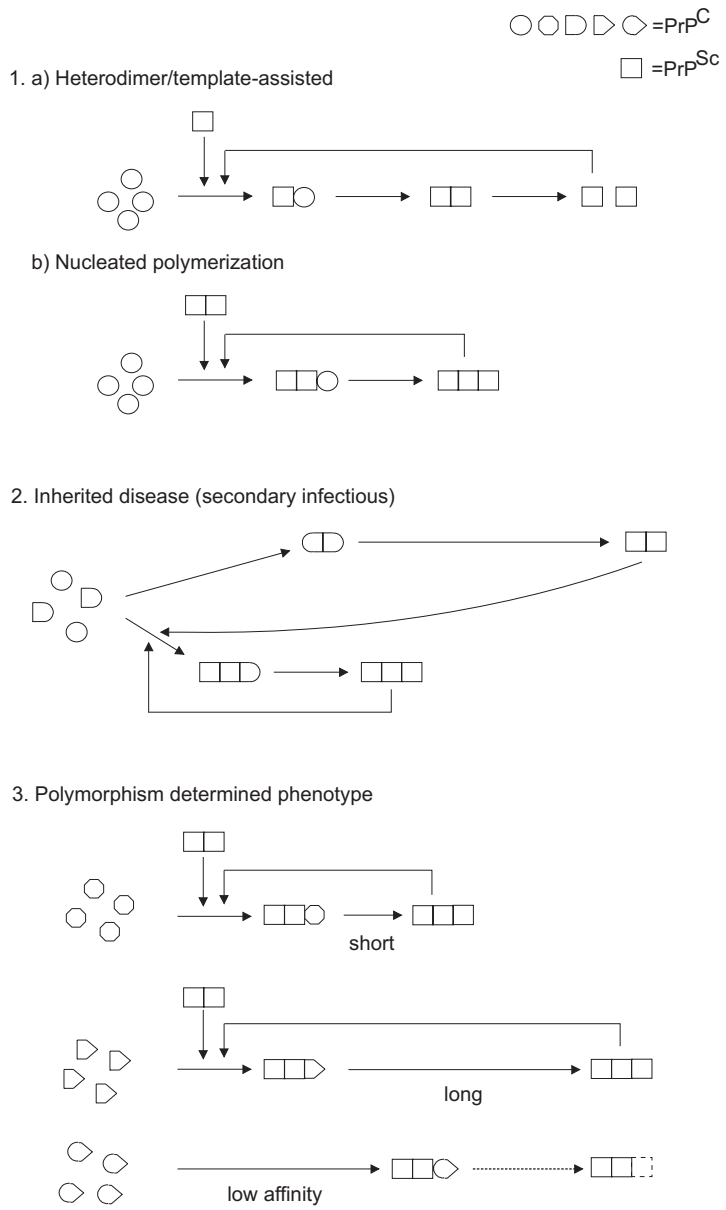


Figure 1. ***Schematic representation of the central events in infectious (panel 1) and inherited (panel 2) forms of TSEs according to the prion hypothesis****. The first panel shows two theoretical mechanistic models for the formation of* PrP^{Sc} *from* PrP^{C}*. The inherited forms shown in panel 2 can also be transmitted causing a secondary infection. Panel 3 demonstrates schematically how polymorphisms in PrP can modulate the phenotypic expression of TSEs (i.e. differences in incubation times). Adapted from Bossers A: Prion diseases, Susceptibility and Transmissibility. PhD Thesis, Utrecht, The Netherlands 1999; with permission.*

Basically two different mechanisms have been proposed for the conversion of PrP^{C} into PrP^{Sc}. In the first mechanism, monomeric PrP^{Sc} acts as a template in a PrP^{Sc}-PrP^{C} heterodimer to form new PrP^{Sc} (Fig. 1 panel 1a) [1, 17]. The second mechanisms is based on the autocatalytic nucleated polymerization in which PrP^{Sc} aggregates induce the conversion of PrP^{C} into PrP^{Sc} (Fig. 1 panel 1b) and infectious PrP^{Sc} molecules only exist in polymeric states [1, 18, 19]. Much experimental evidence supports the validity of the nucleated polymerization mechanism [4, 19-24]. The critical minimal size of the so-called nucleus or ordered aggregate of PrP^{Sc} molecules seems to be at least six PrP molecules to remain associated with conversion activity and TSE infectivity [20, 21]. Others, however, consider the ordered aggregation and polymerization as being an artifact of PrP^{Sc} isolation and favor the template assisted heterodimerisation mechanism [25, 26].

The site at which PrP conversion occurs is at the cell surface and/or in the endocytic pathway towards the lysosomes. Normally PrP^{C} is preferentially linked to cholesterol- and sphingolipid-rich membrane domains ('rafts') via its GPI anchor [27, 28]. Recent studies have indicated that the dissociation of PrP^{C} from these rafts or the insertion of PrP^{Sc} into PrP^{C} containing rafts might be essential for PrP^{Sc} formation [29]. In addition, the association of PrP to other protein or non-protein complexes (chaperone molecules) like bound glycosaminoglycans, or other domain specific properties (pH) might promote or might even be required for the cellular conversion of PrP^{C} into PrP^{Sc} [30-34]. Specific chaperones like GroEL and to a lesser extent Hsp104 have been identified *in vitro* as factors that are able to enhance the conversion process of PrP^{C} into PrP^{Sc} [35] while the association of PrP to glycosaminoglycans seems to be essential for PrP^{Sc} formation in cell culture [33]. However, the conversion of PrP^{C} into protease-resistant forms in a cell-free system is not dependent on such additional factors (Figure 2) [4, 20]. *In vivo* chaperone molecules might also be involved in determining the disease phenotype associated with specific TSE strains. In addition, PrP^{Sc} glycosylation patterns and the dependence of PrP^{Sc} conformation on the binding of copper and zinc may provide a basis for the neuropathological targeting that distinguishes different prion strains [36, 37].

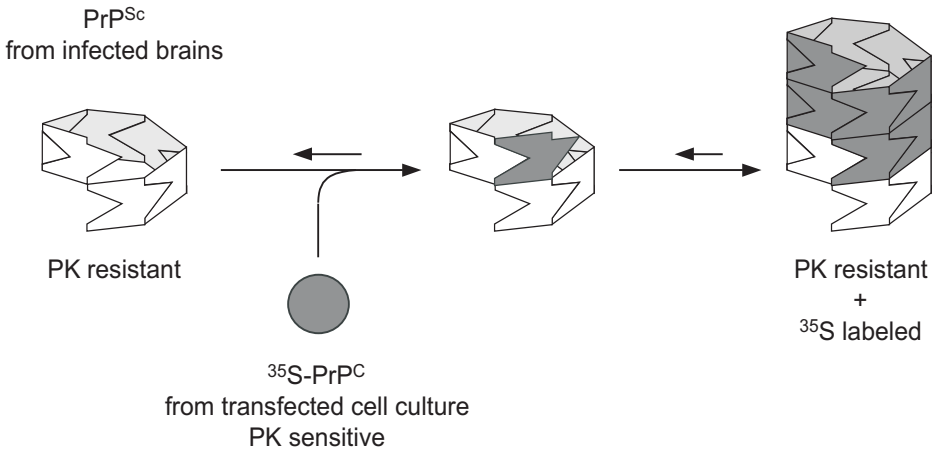


Figure 2. ***Cell-free (in vitro) formation of protease resistant PrP.*** *Immuno-purified and radio-labeled PrP^C (PK-sensitive) is mixed and incubated with partially denatured non-labeled PrP^{Sc} (PK-resistant) isolated from TSE infected brains. After PK digestion of the reaction products, radio labelled PK-resistant PrP can be detected by SDS-PAGE and subsequent auto-radiography or phosphor imaging. Adapted from Bossers A: Prion diseases, Susceptibility and Transmissibility. PhD Thesis, Utrecht, The Netherlands 1999; with permission.*

TSE agent replication in cell culture.

The mouse neuroblastoma (MNB) cells persistently infected with mouse scrapie are probably the most widely used cell culture system to study TSE behavior *in vitro*. This system is capable of maintaining the synthesis of *de novo* mouse scrapie infectivity and accumulates PrP^{Sc} over extended cell passages [38-40]. These scrapie-infected MNB (Sc-MNB) cells were able to produce at least 1 mouse LD50 in less than 50 infected cells [39]. Several mouse-adapted TSE strains could successfully be produced in these MNB cells [41]. The MNB cells also showed species-specificity since these cultures were insensitive to, for instance, hamster scrapie [39] and the Sc-MNB cells readily lost mouse scrapie infectivity after overexpression of heterologous hamster PrP^C [42]. Sc-MNB cells also allowed the determination of the kinetics of PrP^{Sc} formation (including the turn-over of PrP molecules) [43-45]. In addition Sc-MNB cells were used to demonstrate that the PrP conversion occurs after PrP^C has reached the cell-surface and/or translocated into the endocytic pathway towards the lysosomes [46, 47]. Furthermore the Sc-MNB cells were found to be an excellent system for studies towards the inhibition of TSE agent replication. With this system various compounds have been identified which inhibit

PrP^{Sc} formation (see also below paragraph on inhibition and therapeutics) [48, 49]. Potential cytotoxic effects or other negative side-effects of newly developed drugs might be more readily discovered using cell culture systems rather than using cell-free systems. Another advantage of the MNB cell culture system compared to the cell-free system is that it allows cellular (non-)protein chaperone molecules to interact with PrP during its conversion and TSE agent replication. The identified cellular (non-)protein chaperones that assist in TSE agent replication may form potential targets for drug development.

A disadvantage of the Sc-MNB and the MNB cells is that only mouse scrapie or mouse adapted strains of scrapie are able to replicate in these systems. Attempts to introduce TSE agent of different species by overexpression of heterologous PrP in Sc-MNB cells resulted in the loss of scrapie infectivity and PrP conversion [42, 50]. Only a few additional cell lines other than MNB, i.e. hamster brain (HaB) cells [51], rat phaeochromocytoma (PC12) cells [52] and mouse hypothalamic (GT1) cells [53], all of neuronal origin, have been described to be able to replicate mouse scrapie infectivity. The overexpression of heterologous PrP or chimeric PrP molecules did not facilitate the replication of other TSEs in these cell culture systems. Whether the presence of endogenous PrP is the crucial factor interfering with the conversion of overexpressed PrP remains uncertain. Hamster PrP^{C} overexpression in Sc-MNB cells resulted in preventing TSE agent replication in these cells [42]. Our group tried to use Chinese hamster ovary (CHO) cells that have undetectable levels of endogenous hamster PrP^{C} if ovine PrP^{C} is expressed at high levels. Thus far we were unable to generate detectable persistent infections of sheep scrapie in these cells (A. Bossers, unpublished data).

Studies on transmission across species (species barrier) using cell culture systems are very difficult to perform. Only very recently, a rabbit epithelial cell line (Rov) overexpressing sheep PrP has been described that is able to replicate sheep scrapie after passage through mice carrying a sheep PrP transgene [3]. This Rov cell culture system is probably the first cell line in which a TSE agent, other than of mouse origin, can replicate to high titers (Sabuncu and Vilette, Edinburgh UK, personal communication, September 2002). Such new cell lines, and those still in development for other species, are extremely valuable for studying host-agent interactions and species-barriers at the cellular level and may prove more useful in the near future for the development and testing of targeted therapeutics for species other than mouse.

Cell-free conversion of prion proteins.

Basically, cell-free conversion reactions only require the mixing of (purified and radio labeled) normal PrP^{C} with (purified non-labeled) PrP^{Sc} isolated from TSE affected brains (Figure 2). The radio-labeled protease-sensitive PrP^{C} is converted by the protease-resistant PrP^{Sc} into new radio-labeled protease-resistant PrP without the biosynthesis of new macromolecules [4]. The input materials as well as the final conversion products are assayed by SDS-PAGE and auto-radiography / phosphor imaging (example shown in Figure 4).

Initially the system was based on partial denaturing conditions of PrP^{Sc} by chaotropic salts like guanidine-hydrochloride but was later on also adapted to more physiological conditions [35, 54, 55] including intact slices of brains with PrP^{Sc} deposits [8]. The more physiological conditions also allowed studying the effects of potential therapeutics like interfering antibodies, peptides, tetrapyrroles and sulphonated dyes [54, 56-59].

This cell-free system has been used in many different studies towards gaining insight into the underlying molecular mechanisms of TSE agent replication, the existence of TSE strain variation, and to explain inter- and intra-species transmissibilities of prion diseases [4, 7-12, 20, 21]. Guanidine disaggregation and centrifugation studies showed that the cell-free conversion of PrP^{C} into protease-resistant PrP is specifically induced by aggregated forms of PrP^{Sc} (nucleated polymerization as shown in Figure 1 panel 1b) [20, 21]. These studies also showed that especially the aggregated forms of PrP^{Sc} are associated with infectivity [21]. About six polymerized PrP-molecules appears to be the minimal number to induce PrP conversion [20]. Furthermore, the cell-free conversion is generally most efficient when input PrP^{C} and PrP^{Sc} have the same primary amino acid sequence, an observation that accounts for the existence of the barriers for inter- and intra-species transmissions of TSEs [7, 9-13]. Finally, the cell-free system also demonstrated that one and the same PrP^{C} molecule can be converted into at least two different conformational structures underlining the molecular basis of TSE strains [8]. Although the cell-free system represents almost all known biologically relevant parameters associated with TSEs, a 'disadvantage' of the system is that PrP^{Sc} is still required in at least equimolar amounts to PrP^{C} to induce the conversion reaction and that measurements of the *de novo* generated infectivity were still unsuccessful [4, 26, 60].

Recently, however, a new variant on the 'classical' cell-free system has been reported; the protein-misfolding cyclic amplification (PMCA) in which PrP conversion was enhanced by repeated sonication of hamster brain homogenates [6]. In contrast to the 'classical'

cell-free conversion this new system only required minute amounts of PrP^{Sc} but a high molar excess of non-purified PrP^{C} from brain to trigger the conversion reaction. In addition the PMCA reaction also requires additional cellular factors since highly purified fractions of PrP (as used in the 'classical' cell-free system) are incompatible with this system resulting in no detectable PrP conversion. Whether the PMCA system reflects as many biological relevant properties of TSEs as the 'classical' cell-free system and whether *de novo* infectivity is generated remains to be established. Awaiting infectivity assays that are still in progress, preliminary experiments have demonstrated a additional unique feature of the PMCA: demonstrated autocatalytic properties, i.e. newly formed PK-resistant PrP can act as a template to induce subsequent PMCA reactions (Kretzschmar H.A. *et al.*, TSE2002, Edinburgh UK, September 16-18, 2002).

Strain variation.

Prion strain variation is an important phenomenon that determines differences in pathology and differences in inter- and intra-species transmission efficiencies of TSEs, independent of the host PrP amino acid sequences. Strains of TSE agent can be distinguished from each other on the basis of species tropism, clinical disease, incubation period, neuropathological manifestations, PrP^{Sc} distribution in brain tissue, and sometimes also on basis of biochemical and biophysical differences. Different TSE strains can be stably propagated within one and the same inbred host. In the context of the protein-only hypothesis this requires that the propagation of the strain specific properties must be mediated by stable structural variations in PrP^{Sc} independent of the host-encoded PrP amino acid sequence. Indeed several studies like conformational dependent immunoassays [61, 62], biochemical denaturation and protease sensitivity assays [63], or protease digestion and infrared spectroscopy [64] indicate that the biological properties of TSE strains may be enciphered in the conformation of PrP^{Sc}. The existence of different conformational structures for PrP^{Sc} and the copying of these structures to recruited PrP^{C} molecules is an essential prerequisite for the protein-only hypothesis to explain strain-specific properties.

Two molecularly well-characterized strains are the hamster adapted strains of transmissible spongiform encephalopathy (TME), hyper (HY) and drowsy (DY) [65]. These two strains differ in their phenotypic expression in hamsters but also have distinct PrP properties [66]. PrP^{Sc} isolated from HY-TME infected isogenic hamster brains is biochemically distinct from DY-PrP^{Sc} [64, 65]. The two isoforms can readily be distinguished from

each other by SDS-PAGE after limited proteinase-K (PK) digestion (molecular weight difference of about 1-2 kDa). PK hydrolysis produces different fragments from the N-terminal part of PrP^{Sc}, indicating the existence of distinct conformational structures.

Experiments using these two TME strains showed that the strain specific properties of the agent could not only be propagated in inbred hosts *in vivo* [65, 66] but could also be propagated non-genetically *in vitro* (Figure 3) using the cell-free system [8]. One sample of radio labeled hamster PrP^{C} could be converted into two distinct protease-resistant forms of PrP (having the same biochemical differences as the HY or DY PrP^{Sc} that was used to induce the reaction). The efficiencies of those conversion reactions also reflected the phenotypic differences of these TME strains when transmitted to hamsters *in vivo*. These results show that self-propagation of PrP^{Sc} with distinct three-dimensional structures is the molecular basis of prion strain-specificity and that the 3D structure itself is the most probable 'blueprint' for strain specific properties.

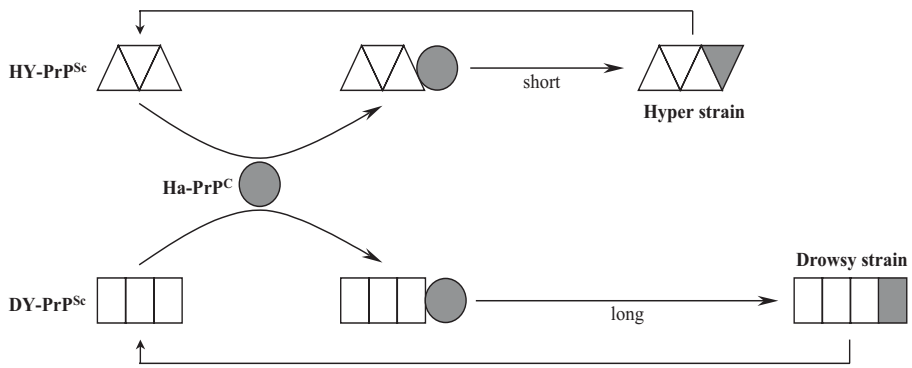


Figure 3. ***Non-genetic propagation of the hamster adapted hyper (HY) and drowsy (DY) strains of TME.*** *HY-PrP^{Sc}, DY-PrP^{Sc}, and hamster PrP^{C} have all the same primary amino acid sequence but differ in three dimensional conformation. The conformation of HY-PrP^{Sc} or DY-PrP^{Sc} is 'inherited' by the one and the same hamster-PrP^{C} molecule. From Bossers A: Prion diseases, Susceptibility and Transmissibility. PhD Thesis, Utrecht, The Netherlands June 10 1999; with permission.*

Polymorphisms in PrP determine inter- and intra-species transmissibilities.

Transmission of TSEs between hosts of the same species is in general more efficient than transmissions between hosts of different species. The barrier to inter-species transmissions often results in incomplete transmissions, complete resistance to transmission (as found in PrP knockout mice) or in increased incubation periods. Subsequent passages in the same isogenic species occur with much higher frequency and shortened incubation periods [67, 68]. The amino acid sequence homology between the PrP^{Sc} constituent of the agent and PrP^{C} of the host is an important parameter in determining the level of the species-barrier. The position and nature of the polymorphisms are critical for determining the strength of these barriers.

Inter-species polymorphism barriers.

Not only experiments with transgenic mice but also studies with *in vitro* systems (cell culture or cell-free conversion assays) have indicated that the PrP sequence of the host strongly influences the susceptibility for TSEs. In addition, the PrP sequence of the donor (PrP^{Sc}) also influences the transmission efficiency to other species [38, 69, 70]. For instance, the species barrier between mouse and hamster, which only differ in ten amino acids in the primary PrP amino acid sequence, has extensively been studied *in vivo* and *in vitro*. Normally there is an almost absolute species-barrier for the cross-species transmission of hamster scrapie to mice while mouse scrapie can be transmitted to hamsters with reduced efficiency. In case of hamster scrapie transmission to mice, this barrier could be overcome solely by introducing a hamster PrP transgene into recipient mice (i.e. express hamster PrP as well as mouse PrP). Strikingly the prions produced by these transgenic mice were either hamster or mouse but were identical to the ones used for inoculation.

Cell-free studies using hamster and mouse PrP^{C} in combination with either hamster or mouse PrP^{Sc} reproduced these *in vivo* observed transmission phenomena [7, 71]. Mouse PrP^{Sc} converted mouse PrP^{C} most efficiently while hamster PrP^{C} was poorly converted. Inversely, hamster PrP^{Sc} converted hamster PrP^{C} most efficiently and was almost unable to convert mouse PrP^{C} [7]. The regions of PrP^{C} and PrP^{Sc} involved in determining species-barriers are probably the regions that are crucial for their interaction. This region was roughly determined for hamster and mouse by chimeric hamster/mouse PrP variants in transgenic mice as well as *in vitro* and the crucial region turned out to be approximately between codons 139 and 170. *In vitro* and *in vivo* studies with several

species have indicated that high homology between PrP^{C} and PrP^{Sc} molecules at amino acid positions 90-225 is preferred for efficient PrP conversion and subsequent disease development [7, 70, 72-75].

Not only complete regions have shown to be involved in determining species barriers but also changes of a single amino acid residue can determine a species-barrier (inter-species transmission efficiency). This has, for instance, been demonstrated by studies of the hamster-mouse species barrier *in vitro* [76]. These species have very similar PrP amino acid sequences, but still encounter a high species-barrier. In a series of experiments using chimeric hamster-mouse PrP molecules in the cell-free system and in Sc-MNB cells, this hamster-mouse species-barrier effect was found to be strongly influenced by homology in a region between amino acids 112 and 170 of PrP [7, 76]. Cell-free PrP conversion experiments using various mouse PrP^{C} variants and hamster PrP^{Sc} showed that especially homology at position 155 was critical [71]. These observations stress that the nature and location of a specific polymorphism in PrP could be most important for determining transmission barriers.

After the cell-free system had shown species- and strain-specificity [7, 8], the next logical step was to use the system for assessment of the species barriers. Therefore, the species-specificity of the cell-free conversion as found by Kocisko *et al.* 1994 for hamster and mouse was further extended by demonstrating that many known species-barriers of *in vivo* transmissions could be reflected within the cell-free conversion reaction. The study of Raymond *et al.* 1997 clearly showed that the cell-free system demonstrated many known barriers to interspecies transmission. Thereby the cell-free system gained predictive value that allowed the molecular assessments of several unknown (or immeasurable) species barriers. Thus far the cell-free conversion assays were used to analyze species barriers among animals and humans (e.g. BSE, CWD) [11, 12]. To which extent the various cell-free conversion efficiencies can be taken as a quantitative measure for species-barriers is still unclear, largely due to the lack of quantitative *in vivo* titration data within the various species. Based on the available information however, it seems that the log of the infectivity titer determined after intracerebral inoculation of PrP^{Sc} is roughly proportional to the relative cell-free conversion efficiency on a linear scale [12].

Intra-species polymorphism barriers.

Several single amino acid changes within a single species are known to determine the phenotypic expressions of TSEs (reviewed by Young *et al.* 1999 [77]). The most striking example is probably the behavior of the PrP haplotype resulting from a polymorphism

at residue 178 and the methionine/valine polymorphism at residue 129 in humans. If codon 178 encodes an asparagine (N) and codon 129 a methionine (M) the patient develops fatal familial insomnia (FFI), while patients having N at codon 178 and valine (V) at codon 129 develop Creutzfeldt-Jakob disease (CJD) [78]. The PrP codon 129 polymorphism in humans is also associated with differences in incubation periods of iatrogenic forms of CJD [79, 80] and probably determines the susceptibility for variant CJD (vCJD) development since all of vCJD patients are codon 129M homozygotes [81]. Results of cell-free conversion experiments confirm that humans homozygous for 129M are probably more susceptible to BSE than humans homozygous for 129V [11].

In the PrP gene of sheep several polymorphisms have been detected that are associated with scrapie susceptibility [82-88]. The allelic variant having the alanine (A) to V substitution at codon 136, is significantly associated with a high susceptibility to scrapie and short survival times of scrapie-affected sheep in many different breeds [70, 84, 87-90]. In contrast, the allelic variant having a codon 171 glutamine (Q) to arginine (R) substitution is associated with resistance to natural and experimental infections with scrapie and BSE in probably all sheep breeds [70, 82-84, 87, 89]. In breeds where the codon 136V allele is rare or absent the 'wildtype' PrP allelic variant (136A and 171Q) is associated with increased scrapie susceptibility, but with a lower penetrance than found for the 136V allele [90, 91].

Cell-free conversion studies using several allelic variants of sheep PrP^{C} and PrP^{Sc} have demonstrated that single polymorphisms in sheep PrP can modulate disease development [9-11]. Polymorphisms present in either sheep PrP^{C} or sheep PrP^{Sc} had dramatic effects on the cell-free conversion efficiencies (see Figure 4 and 5). These efficiencies correlated well with their known association with scrapie susceptibility [9, 10, 12]. The PrP^{C_136V} variant was most efficiently converted by PrP^{Sc_136V} (Figure 4 lane 7), wildtype (wt) PrP^{C} was converted with intermediate efficiency by PrP^{Sc_136V} (Figure 4 lane 8), while the variant PrP^{C_171R}, associated with resistance *in vivo*, was almost unconvertible by PrP^{Sc_136V} (Figure 4 lane 9). In addition, Rov cells expressing sheep PrP^{C_136V} at high levels were very sensitive to sheep prion transmission and replicated the agent to high titers. In contrast, no evidence of infection could be found in Rov cells expressing similar levels of the PrP^{C_171R} variant that is linked to resistance (Sabuncu and Vilette, Edinburgh UK, personal communication, September 2002). In conclusion, the results of these two systems indicate that the observed variations in sheep scrapie incubation periods are largely encrypted within the PrP genotypes.

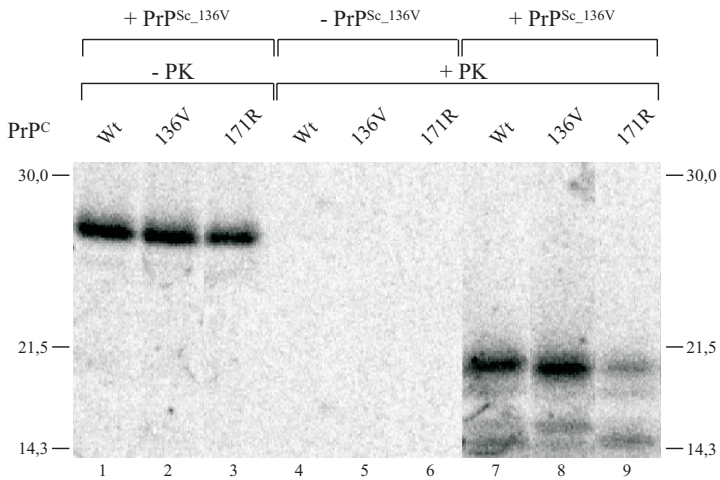


Figure 4. ***Example phosphor-image of a SDS-PAGE showing the cell-free conversion reactions of three different non-glycosylated sheep PrP^{C} variants into PK-resistant forms induced by sheep PrP^{Sc_136V}.*** *Lanes 1-3 show approximately 1/10 of the input of PrP^{C} in the various conversion reactions. Incubation of these variants without PrP^{Sc} under cell-free conditions revealed no spontaneous protease resistant PrP formation after PK digestion (lanes 4-6). Different amounts of protease-resistant PrP were formed however, induced by addition of partially denatured exogenous PrP^{Sc} (lanes 7-9). PK digestion removes about 6 kDa from the N-terminal part of PrP when converted into protease resistant forms (compare lanes 1-3 with lanes 7-9). Molecular mass markers (kDa) are indicated. Data from Bossers, A., de Vries, R. & Smits, M. A. (2000). Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. Journal of Virology 74, 1407-14.*

Since the cell-free conversion efficiencies reflected inter- and intra-species transmission efficiencies, the system has also been used to asses the potential effects of certain less frequent or rare polymorphisms in sheep PrP which could not significantly be associated *in vivo* with any disease phenotype yet. Six of such additional variants were therefore tested in the cell-free system using the three, at that time available, homozygous types of sheep PrP^{Sc}; PrP^{Sc_wt}, PrP^{Sc_136V}, and PrP^{Sc_171H} (Figure 5) [10]. The normalized conversion efficiencies clearly show that each type of PrP^{Sc} had its own readily distinguishable 'conversion profile' (differential conversion efficiencies for the various PrP^{C} variants). Since this set of natural sheep PrP variants is capable of distinguishing three types of PrP^{Sc} it might be possible in the near future to construct a set of natural and/or artificial PrP^{C} variants that can be used for differentiation of various (sheep) TSE isolates, including the from cattle BSE derived isolate that potentially crossed the species barrier (back) into sheep.

The exact mechanism by which the polymorphisms influence conversion activity and disease phenotype is still unclear. Cell-free conversion studies have shown that the polymorphisms modulate the conversion process of PrP^{C} into protease resistant PrP in a very specific and reproducible way. Structural studies in PrP^{C} and PrP^{Sc} may in the future provide more insight in the effects that these polymorphisms may have at the molecular level. Studies into the thermal unfolding pathways of several polymorphic sheep PrP^{C} variants have shown that in general variants associated with increased susceptibility have unfolding properties that are different for variants associated with decreased susceptibility [92]. Basically the effects of the polymorphisms are predicted to modulate events at several stages of the PrP conversion as indicated in the last panel of Figure 1. The various PrP^{C} molecules may bind to the PrP^{Sc} with the same efficiency but might convert differentially into protease resistant forms and/or the PrP^{C} molecules might bind differentially to the PrP^{Sc} and/or its chaperones. Recent preliminary unpublished results of sheep PrP^{C}-PrP^{Sc} binding studies, analogous to those performed using PrP of hamster and mouse [55], suggest that the various sheep PrP^{C} variants all bind to the sheep PrP^{Sc}.

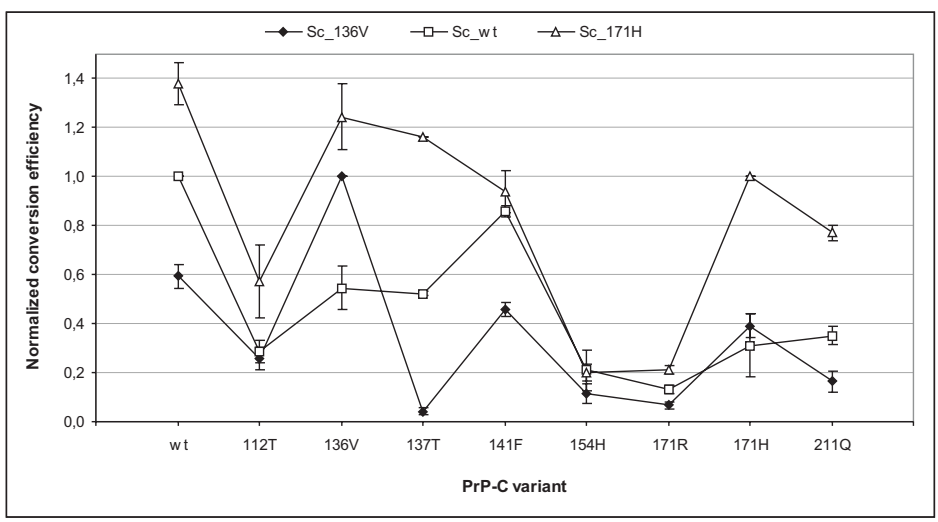


Figure 5. ***Normalized cell-free conversion efficiencies of nine different natural sheep PrP^{C} variants incubated with three different types of sheep PrP^{Sc}.*** *Within each type of PrP^{Sc} used, the relative conversion efficiencies of nine PrP^{C} variants are shown compared to the homologous conversion reaction (i.e. PrP^{Sc_wt}/PrP^{C_wt}, $PrP^{Sc\_136V}/PrP^{C\_136V}$, and $PrP^{Sc\_171H}/PrP^{C\_171H}$; which have been set at unity (1.0)). Mean normalized conversion efficiencies (± S.E.M.) are indicated. Data from Bossers, A., de Vries, R. & Smits, M. A. (2000). Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. Journal of Virology 74, 1407-14*

Figure 6 shows the results of such a preliminary experiment of the binding of three sheep PrP^{C} variants (wt, 136V, and 171R) to wildtype sheep PrP^{Sc} (phylogenetic wildtype in sheep has been defined as the variant from which all other variants can be made by only a single mutation. For sheep being 136A, 154R, and 171Q). In this experiment radio labeled and immuno-precipitated PrP^{C} was incubated for two days with PrP^{Sc} isolated from sheep brain. Aggregates of PrP^{Sc} (and potentially bound PrP^{C}) were collected by low speed centrifugation (20 min. 14,000 x g at 4°C). Pellet and supernatant fractions were separated and analyzed by sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE) and phosphor imaging. The three PrP^{C} variants all seem to bind to high degree to PrP^{Sc} since almost all PrP^{C} signal is collected in the pellet fraction (Figure 6 lanes 1, 5, and 9) while the supernatant becomes devoid of PrP^{C} (Figure 6 lanes 2, 6, and 10). In contrast, in case where PrP^{C} was incubated without PrP^{Sc} almost all PrP^{C} signal remained in the supernatant (Figure 6 lanes 4, 8, and 12). The preliminary data show that using guanidine-free conditions the 171R variant of sheep PrP^{C} is able to bind efficiently to several types of sheep PrP^{Sc} (results of wildtype sheep PrP^{Sc} are shown only in figure 6) although the exact binding kinetics should still be determined. Roughly the 171R PrP^{C} binding efficiency to wildtype PrP^{Sc} is comparable to that of the 136V PrP^{C} variant (compare Figure 6 lanes 1 and 2 with 9 and 10). Whether all the PrP^{C} variants bind specifically to the nucleation/conversion site(s) of PrP^{Sc} or nonspecific binding to PrP^{Sc} occurs, is still unclear. Competition assays and/or PrP binding/dissociation studies with surface plasmon resonance techniques may help to address these questions. The preliminary results of the binding experiments suggest that the key mechanism underlying the differences in conversion efficiencies of various sheep PrP^{C} variants seem not to be determined by differential binding efficiencies of PrP^{C} to PrP^{Sc}.

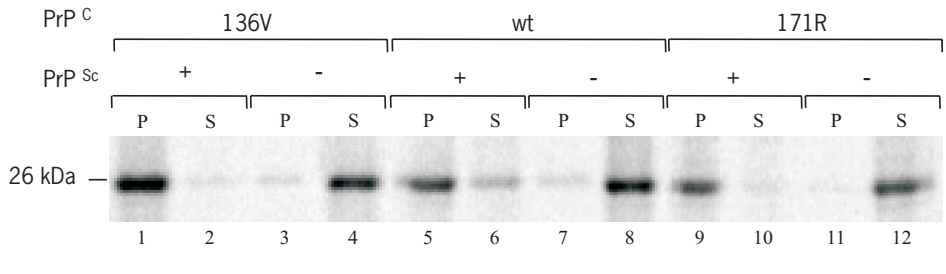


Figure 6. ***Example of binding experiment of radio-labeled sheep PrP^{C} to non-labeled sheep PrP^{Sc}.*** *Three polymorphic variants of sheep PrP^{C} were incubated with wildtype sheep PrP^{Sc} for 2 days after which bound PrP^{C} (pellet (P)) was separated from unbound PrP^{C} (supernatant (S)) by low speed centrifugation. Radio-labeled PrP^{C} of about 26 kDa was visualised by SDS-PAGE and phosphor imaging.*

Prospects for TSE therapeutics determined by inhibition of in vitro prion protein conversion.

Since PrP conversion and the accumulation of PrP^{Sc} are central pathogenetic events in TSEs, one of the major approaches for TSE therapy is the inhibition of PrP conversion and/or accumulation. Although several inhibitors have been identified, none has thus far proven to be effective for the treatment of clinical TSE. The cell culture and cell-free systems provide a direct tool to study and find prion conversion inhibitors. The systems are in general rapid, the circumstances are not as complex as in animal hosts and thereby more easy to control or modify, and the *in vitro* systems are more suitable for medium to high-throughput testing of panels of potential drugs.

Using *in vitro* systems several compounds have been identified that inhibit PrP conversion and/or destabilize PrP^{Sc}. These compounds include β-sheet breaker peptides [93], peptides binding to the potential conversion site of PrP^{C} or PrP^{Sc} [56, 94], sulfated polysaccharides [95], branched polyamines [96], tetrapyrroles [58, 97], Congo red related dyes [59], lysosomotropic agents and cysteine protease inhibitors [98]. Although some of these compounds have blocked PrP conversion *in vitro* and also prolonged the survival time of animals infected by TSEs, presently no practical administrable drugs are available for treatment of TSEs.

Other lines currently under investigation to inhibit PrP conversion are for instance the generation of (temporarily) PrP knockouts by specific chimeraplasts (Metz RA, CHI meeting on transmissible spongiform encephalopathies, Washington USA, October 27-28, 1999), by anti-sense inhibition of PrP^{C} expression, or by the use of conversion resistant PrP molecules. In addition it is known that heterologous PrP molecules can efficiently interfere with prion replication in transgenic mice and also in *in vitro* systems [42, 50, 55]. Heterologous expression of hamster PrP^{C} in scrapie infected MNB cells cures the scrapie infection [42]. Furthermore, homozygosity for PrP is a risk factor for TSE development in humans and animals. For example, humans who are M/V heterozygous for PrP at codon 129 have prolonged incubation periods over the 129M/M or 129V/V homozygotes [79, 80]. Sheep heterozygous for the 171R PrP allele are also largely protected to natural scrapie development independent of the presence of one normal 'susceptible' PrP allele [70, 82, 84, 87, 89]. Since heterologous PrP is able to interfere with PrP conversion it might form a component of future drugs. Administration of the potential drugs and the delivery to the site of action is an additional problem encountered *in vivo*. Gene therapy strategies using site or tissue specific (viral) delivery systems might solve this problem.

In vitro systems provide efficient tools to rapidly measure any interference of heterologous PrP variants [42, 55]. In addition, natural or artificial polymorphic PrP variants could be identified *in vitro* that are inconvertible but that are still able to bind PrP^{Sc} with high affinity. Such PrP variants might in (temporarily or inducible) gene therapy prolong the TSE survival time (Figure 7) by blocking PrP conversion. An advantage of using a polymorphism-based strategy would be that host-encoded PrP can be used as basis for the polymorphic mutant thereby avoiding potential host immune responses to the drug. Prerequisites of therapeutic polymorphic variants would be that they are stable, that they do not form spontaneous PrP^{Sc}, that they bind with high affinity to PrP^{Sc} (at best higher than the endogenous PrP^{C} binds to PrP^{Sc}), and that they thereby block the PrP conversion process. Most of these properties can readily be measured using cell-free or cell culture systems.

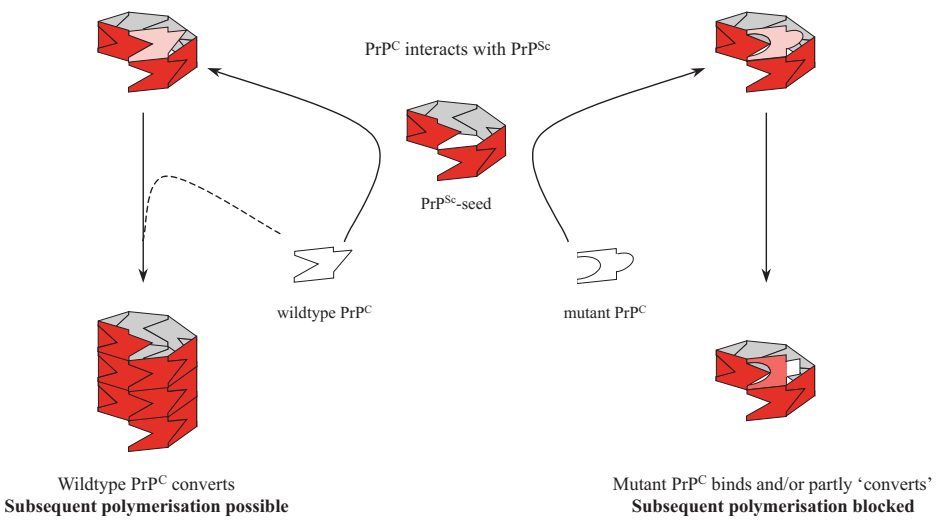


Figure 7. ***Hypothetical representation of the conversion of PrP and the potential inhibition by mutant PrP^{C} molecules.*** *PrP^{C} is efficiently converted into PK-resistant PrP by a PrP^{Sc} seed ('infectious particle') which allows the subsequent polymerization (TSE 'replication'). However, if mutant PrP^{C} binds to the PrP^{Sc} seed (ideally) with higher or equal affinity as wildtype PrP^{C}, the mutant PrP^{C} might be able to block the resulting nucleation site and the subsequent polymerization (replication). Adapted from Bossers A: Prion diseases, Susceptibility and Transmissibility. PhD Thesis, Utrecht, The Netherlands 1999; with permission.*

Concluding remarks.

The *in vitro* conversion techniques either cell-free or in cell culture systems, have provided us with tools to adequately study the underlying mechanism of TSEs, namely PrP conversion. These systems have also provided the tools to more easily study the inter- and intra-species transmissibilities of TSEs. Finally these systems might also assist in the discovery of TSE therapeutic strategies and the development of extremely sensitive TSE detection techniques.

Thus far the cell culture systems are still limited to two different species while the cell-free system has the advantage that virtually all forms of TSE can be studied including those to which experimental transmissions cannot be done (i.e. to humans). The cell culture systems however, have the advantage that they generate *de novo* TSE infectivity. Studies using cell cultures also take into account several cellular factors in addition to PrP that might be involved in replication of TSEs. Although the *in vitro* systems provided us with accurate tools to study TSE agent parameters, we still need to keep in mind that these systems mainly or only focus on the molecular processes of PrP conversion. Other factors, i.e. host genetic factors [99] that for example determine the differential uptake of the TSE agent from the environment, play additional roles in susceptibility of hosts for TSEs and on the transmission of the disease between individuals.

References.

1. Griffith, J.S., *Self-replication and scrapie.* Nature, 1967. 215(105): p. 1043-4.
2. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. 216(4542): p. 136-44.
3. Vilette, D., et al., *Ex vivo propagation of infectious sheep scrapie agent in heterologous epithelial cells expressing ovine prion protein.* Proc Natl Acad Sci U S A, 2001. 98(7): p. 4055-9.
4. Kocisko, D.A., et al., *Cell-free formation of protease-resistant prion protein.* Nature, 1994. 370(6489): p. 471-4.
5. Saborio, G.P., et al., *Cell-lysate conversion of prion protein into its protease-resistant isoform suggests the participation of a cellular chaperone.* Biochem Biophys Res Commun, 1999. 258(2): p. 470-5.
6. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. 411(6839): p. 810-3.
7. Kocisko, D.A., et al., *Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier.* Proc Natl Acad Sci U S A, 1995. 92(9): p. 3923-7.
8. Bessen, R.A., et al., *Non-genetic propagation of strain-specific properties of scrapie prion protein.* Nature, 1995. 375(6533): p. 698-700.
9. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.
10. Bossers, A., R. de Vries, and M.A. Smits, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol, 2000. 74(3): p. 1407-14.
11. Raymond, G.J., et al., *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans.* Nature, 1997. 388(6639): p. 285-8.
12. Raymond, G.J., et al., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo J, 2000. 19(17): p. 4425-30.
13. Kimberlin, R.H., C.A. Walker, and G.C. Millson, *Letter: Interspecies transmission of scrapie-like diseases.* Lancet, 1975. 2(7948): p. 1309-10.
14. Hegde, R.S., et al., *A transmembrane form of the prion protein in neurodegenerative disease.* Science, 1998. 279(5352): p. 827-34.
15. Stewart, R.S. and D.A. Harris, *Most pathogenic mutations do not alter the membrane topology of the prion protein.* J Biol Chem, 2001. 276(3): p. 2212-20.

16. Lehmann, S. and D.A. Harris, *Two mutant prion proteins expressed in cultured cells acquire biochemical properties reminiscent of the scrapie isoform.* Proc Natl Acad Sci U S A, 1996. 93(11): p. 5610-4.
17. Prusiner, S.B., *Molecular biology of prion diseases.* Science, 1991. 252(5012): p. 1515-22.
18. Jarrett, J.T. and P.T. Lansbury, Jr., *Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?* Cell, 1993. 73(6): p. 1055-8.
19. Come, J.H., P.E. Fraser, and P.T. Lansbury, Jr., *A kinetic model for amyloid formation in the prion diseases: importance of seeding.* Proc Natl Acad Sci U S A, 1993. 90(13): p. 5959-63.
20. Caughey, B., et al., *Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state.* Chem Biol, 1995. 2(12): p. 807-17.
21. Caughey, B., et al., *Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies.* J Virol, 1997. 71(5): p. 4107-10.
22. Gajdusek, D.C., *Transmissible and non-transmissible amyloidoses: autocatalytic post-translational conversion of host precursor proteins to beta-pleated sheet configurations.* J Neuroimmunol, 1988. 20(2-3): p. 95-110.
23. Kocisko, D.A., P.T. Lansbury, Jr., and B. Caughey, *Partial unfolding and refolding of scrapie-associated prion protein: evidence for a critical 16-kDa C-terminal domain.* Biochemistry, 1996. 35(41): p. 13434-42.
24. Lansbury, P.T., Jr. and B. Caughey, *The chemistry of scrapie infection: implications of the 'ice 9' metaphor.* Chem Biol, 1995. 2(1): p. 1-5.
25. McKinley, M.P., et al., *Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis.* J Virol, 1991. 65(3): p. 1340-51.
26. Kaneko, K., et al., *Prion protein (PrP) synthetic peptides induce cellular PrP to acquire properties of the scrapie isoform.* Proc Natl Acad Sci U S A, 1995. 92(24): p. 11160-4.
27. Harris, D.A., *Cellular biology of prion diseases.* Clin Microbiol Rev, 1999. 12(3): p. 429-44.
28. Naslavsky, N., et al., *Sphingolipid depletion increases formation of the scrapie prion protein in neuroblastoma cells infected with prions.* J Biol Chem, 1999. 274(30): p. 20763-71.
29. Baron, G.S., et al., *Conversion of raft associated prion protein to the protease-resistant state requires insertion of PrP-res (PrP(Sc)) into contiguous membranes.* EMBO J, 2002. 21(5): p. 1031-40.
30. Naslavsky, N., et al., *Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform.* J Biol Chem, 1997. 272(10): p. 6324-31.
31. Snow, A.D., et al., *Sulfated glycosaminoglycans in amyloid plaques of prion diseases.* Acta Neuropathol, 1989. 77(4): p. 337-42.

32. Brimacombe, D.B., et al., *Characterization and polyanion-binding properties of purified recombinant prion protein.* Biochem J, 1999. 342 Pt 3: p. 605-13.
33. Caughey, B., D. Ernst, and R.E. Race, *Congo red inhibition of scrapie agent replication.* J Virol, 1993. 67(10): p. 6270-2.
34. Telling, G.C., et al., *Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein.* Cell, 1995. 83(1): p. 79-90.
35. DebBurman, S.K., et al., *Chaperone-supervised conversion of prion protein to its protease-resistant form.* Proc Natl Acad Sci U S A, 1997. 94(25): p. 13938-43.
36. Wadsworth, J.D., et al., *Strain-specific prion-protein conformation determined by metal ions.* Nat Cell Biol, 1999. 1(1): p. 55-9.
37. Collinge, J., *Prion diseases of humans and animals: their causes and molecular basis.* Annu Rev Neurosci, 2001. 24: p. 519-50.
38. Race, R.E., et al., *Analyses of frequency of infection, specific infectivity, and prion protein biosynthesis in scrapie-infected neuroblastoma cell clones.* J Virol, 1988. 62(8): p. 2845-9.
39. Race, R.E., L.H. Fadness, and B. Chesebro, *Characterization of scrapie infection in mouse neuroblastoma cells.* J Gen Virol, 1987. 68 (Pt 5): p. 1391-9.
40. Butler, D.A., et al., *Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins.* J Virol, 1988. 62(5): p. 1558-64.
41. Nishida, N., et al., *Successful transmission of three mouse-adapted scrapie strains to murine neuroblastoma cell lines overexpressing wild-type mouse prion protein.* J Virol, 2000. 74(1): p. 320-5.
42. Priola, S.A., et al., *Heterologous PrP molecules interfere with accumulation of protease-resistant PrP in scrapie-infected murine neuroblastoma cells.* J Virol, 1994. 68(8): p. 4873-8.
43. Caughey, B., et al., *In vitro expression in eukaryotic cells of a prion protein gene cloned from scrapie-infected mouse brain.* Proc Natl Acad Sci U S A, 1988. 85(13): p. 4657-61.
44. Borchelt, D.R., et al., *Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells.* J Cell Biol, 1990. 110(3): p. 743-52.
45. Caughey, B. and G.J. Raymond, *The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive.* J Biol Chem, 1991. 266(27): p. 18217-23.
46. Caughey, B., *Cellular metabolism of normal and scrapie-associated forms of PrP.* Sem Virol, 1991: p. 189-196.
47. Caughey, B., et al., *N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state.* J Virol, 1991. 65(12): p. 6597-603.
48. Head, M.W. and J.W. Ironside, *Inhibition of prion-protein conversion: a therapeutic tool?* Trends Microbiol, 2000. 8(1): p. 6-8.

49. Priola, S.A., *Prion protein and species barriers in the transmissible spongiform encephalopathies.* Biomed Pharmacother, 1999. 53(1): p. 27-33.
50. Holscher, C., H. Delius, and A. Burkle, *Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation.* J Virol, 1998. 72(2): p. 1153-9.
51. Taraboulos, A., D. Serban, and S.B. Prusiner, *Scrapie prion proteins accumulate in the cytoplasm of persistently infected cultured cells.* J Cell Biol, 1990. 110(6): p. 2117-32.
52. Rubenstein, R., R.I. Carp, and S.M. Callahan, *In vitro replication of scrapie agent in a neuronal model: infection of PC12 cells.* J Gen Virol, 1984. 65 (Pt 12): p. 2191-8.
53. Schatzl, H.M., et al., *A hypothalamic neuronal cell line persistently infected with scrapie prions exhibits apoptosis.* J Virol, 1997. 71(11): p. 8821-31.
54. Horiuchi, M. and B. Caughey, *Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state.* Embo J, 1999. 18(12): p. 3193-203.
55. Horiuchi, M., et al., *Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers.* Proc Natl Acad Sci U S A, 2000. 97(11): p. 5836-41.
56. Chabry, J., B. Caughey, and B. Chesebro, *Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides.* J Biol Chem, 1998. 273(21): p. 13203-7.
57. Chabry, J., et al., *Species-independent inhibition of abnormal prion protein (PrP) formation by a peptide containing a conserved PrP sequence.* J Virol, 1999. 73(8): p. 6245-50.
58. Caughey, W.S., et al., *Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines.* Proc Natl Acad Sci U S A, 1998. 95(21): p. 12117-22.
59. Demaimay, R., B. Chesebro, and B. Caughey, *Inhibition of formation of protease-resistant prion protein by Trypan Blue, Sirius Red and other Congo Red analogs.* Arch Virol Suppl, 2000(16): p. 277-83.
60. Hill, A.F., M. Antoniou, and J. Collinge, *Protease-resistant prion protein produced in vitro lacks detectable infectivity.* J Gen Virol, 1999. 80 (Pt 1): p. 11-4.
61. Safar, J., et al., *Eight prion strains have PrP(Sc) molecules with different conformations.* Nat Med, 1998. 4(10): p. 1157-65.
62. Safar, J., F.E. Cohen, and S.B. Prusiner, *Quantitative traits of prion strains are enciphered in the conformation of the prion protein.* Arch Virol Suppl, 2000(16): p. 227-35.
63. Peretz, D., et al., *Antibodies inhibit prion propagation and clear cell cultures of prion infectivity.* Nature, 2001. 412(6848): p. 739-43.
64. Caughey, B., G.J. Raymond, and R.A. Bessen, *Strain-dependent differences in beta-sheet conformations of abnormal prion protein.* J Biol Chem, 1998. 273(48): p. 32230-5.

65. Bessen, R.A. and R.F. Marsh, *Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent.* J Virol, 1992. 66(4): p. 2096-101.

66. Bessen, R.A. and R.F. Marsh, *Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy.* J Virol, 1994. 68(12): p. 7859-68.

67. Foster, J.D. and A.G. Dickinson, *The unusual properties of CH1641, a sheep-passaged isolate of scrapie.* Vet Rec, 1988. 123(1): p. 5-8.

68. Pattison, I.H., *The relative susceptibility of sheep, goats and mice to two types of the goat scrapie agent.* Res Vet Sci, 1966. 7(2): p. 207-12.

69. Carlson, G.A., et al., *Primary structure of prion protein may modify scrapie isolate properties.* Proc Natl Acad Sci U S A, 1989. 86(19): p. 7475-9.

70. Goldmann, W., et al., *PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie.* J Gen Virol, 1994. 75 (Pt 5): p. 989-95.

71. Priola, S.A. and V.A. Lawson, *Glycosylation influences cross-species formation of protease-resistant prion protein.* Embo J, 2001. 20(23): p. 6692-9.

72. Priola, S.A., et al., *Prion protein and the scrapie agent: in vitro studies in infected neuroblastoma cells.* Infect Agents Dis, 1994. 3(2-3): p. 54-8.

73. Priola, S.A., et al., *A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP.* J Biol Chem, 1995. 270(7): p. 3299-305.

74. Scott, M.R., et al., *Chimeric prion protein expression in cultured cells and transgenic mice.* Protein Sci, 1992. 1(8): p. 986-97.

75. Telling, G.C., et al., *Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein.* Proc Natl Acad Sci U S A, 1994. 91(21): p. 9936-40.

76. Priola, S.A. and B. Chesebro, *A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells.* J Virol, 1995. 69(12): p. 7754-8.

77. Young, M., et al., *Predicting conformational switches in proteins.* Protein Sci, 1999. 8(9): p. 1752-64.

78. Goldfarb, L.G., et al., *Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism.* Science, 1992. 258(5083): p. 806-8.

79. Collinge, J., M.S. Palmer, and A.J. Dryden, *Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease.* Lancet, 1991. 337(8755): p. 1441-2.

80. Palmer, M.S., et al., *Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease.* Nature, 1991. 352(6333): p. 340-2.

81. Collinge, J., et al., *Prion protein gene analysis in new variant cases of Creutzfeldt-Jakob disease.* Lancet, 1996. 348(9019): p. 56.

82. Belt, P.B., et al., *Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie.* J Gen Virol, 1995. 76 (Pt 3): p. 509-17.

83. Belt, P.B.G.M., et al., *PrP allelic variants associated with natural scrapie.*, in *Bovine Spongiform Encephalopathy; The BSE dilemma*, C.J. Gibbs Jr., Editor. 1996, Springer: New York. p. 294-305.

84. Bossers, A., et al., *PrP genotype contributes to determining survival times of sheep with natural scrapie.* J Gen Virol, 1996. 77 (Pt 10): p. 2669-73.

85. Goldmann, W., et al., *Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene.* J Gen Virol, 1991. 72 (Pt 10): p. 2411-7.

86. Goldmann, W., et al., *Two alleles of a neural protein gene linked to scrapie in sheep.* Proc Natl Acad Sci U S A, 1990. 87(7): p. 2476-80.

87. Hunter, N., et al., *Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes.* Arch Virol, 1996. 141(5): p. 809-24.

88. Laplanche, J.L., et al., *PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis.* Genomics, 1993. 15(1): p. 30-7.

89. Clouscard, C., et al., *Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie.* J Gen Virol, 1995. 76 (Pt 8): p. 2097-101.

90. Hunter, N., et al., *The association of a codon 136 PrP gene variant with the occurrence of natural scrapie.* Arch Virol, 1994. 137(1-2): p. 171-7.

91. Westaway, D., et al., *Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie.* Genes Dev, 1994. 8(8): p. 959-69.

92. Rezaei, H., et al., *Amyloidogenic unfolding intermediates differentiate sheep prion protein variants.* J Mol Biol, 2002. 322(4): p. 799-814.

93. Soto, C., et al., *Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides.* Lancet, 2000. 355(9199): p. 192-7.

94. Horiuchi, M., et al., *Inhibition of interactions and interconversions of prion protein isoforms by peptide fragments from the C-terminal folded domain.* J Biol Chem, 2001. 276(18): p. 15489-97.

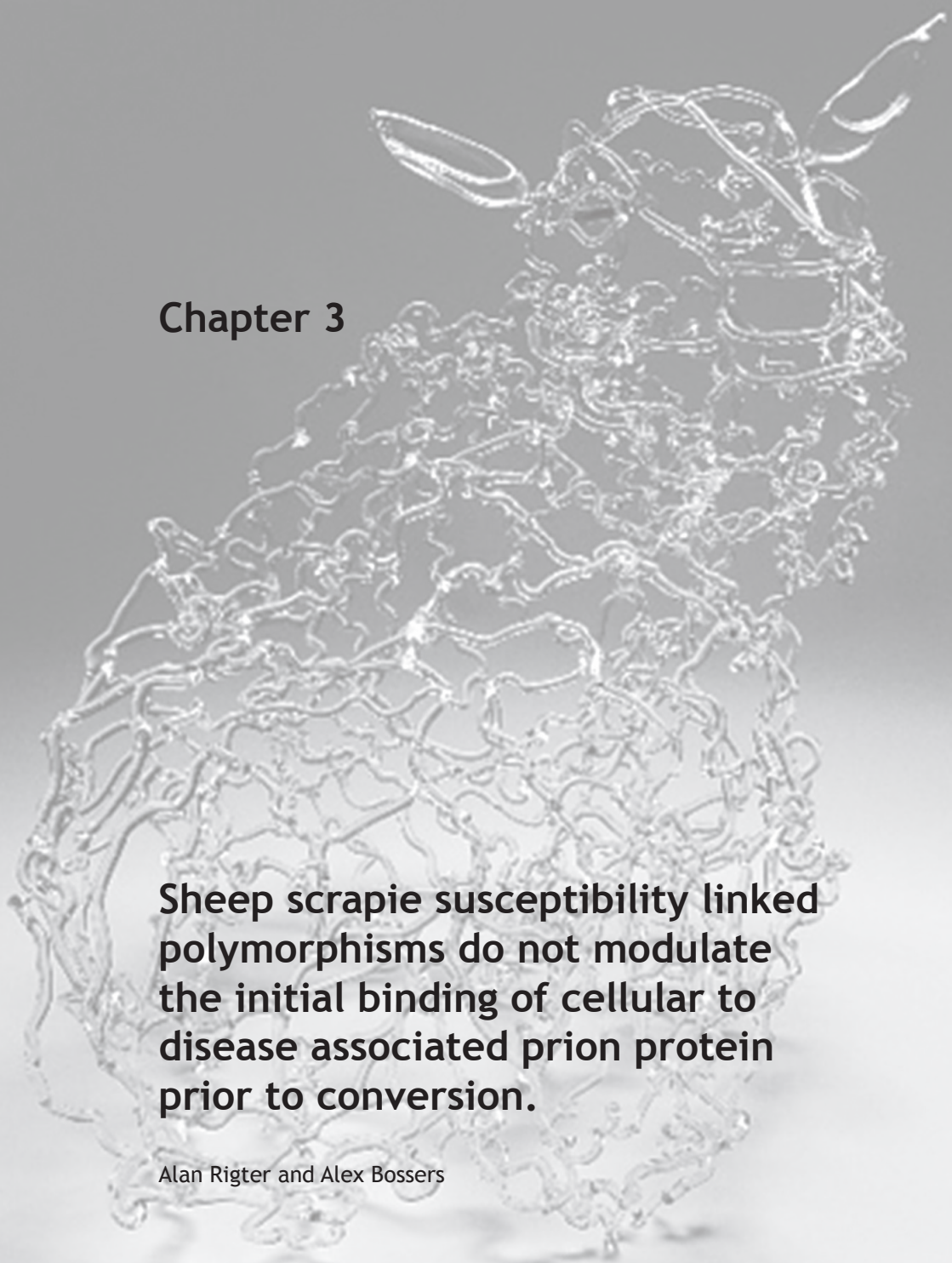
95. Ladogana, A., et al., *Sulphate polyanions prolong the incubation period of scrapie-infected hamsters.* J Gen Virol, 1992. 73 (Pt 3): p. 661-5.

96. Supattapone, S., et al., *Branched polyamines cure prion-infected neuroblastoma cells.* J Virol, 2001. 75(7): p. 3453-61.

97. Priola, S.A., A. Raines, and W.S. Caughey, *Porphyrin and phthalocyanine antiscrapie compounds.* Science, 2000. 287(5457): p. 1503-6.

98. Doh-Ura, K., T. Iwaki, and B. Caughey, *Lysosomotropic agents and cysteine protease inhibitors inhibit scrapie-associated prion protein accumulation.* J Virol, 2000. 74(10): p. 4894-7.

99. Lloyd, S.E., et al., *Identification of multiple quantitative trait loci linked to prion disease incubation period in mice.* Proc Natl Acad Sci U S A, 2001. 98(11): p. 6279-83.



Chapter 3

Sheep scrapie susceptibility linked polymorphisms do not modulate the initial binding of cellular to disease associated prion protein prior to conversion.

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Sheep scrapie susceptibility linked polymorphisms do not modulate the initial binding of cellular to disease associated prion protein prior to conversion.

Alan Rigter and Alex Bossers

Abstract.

Conversion of host-encoded protease sensitive cellular prion protein (PrP^{C}) into the scrapie associated protease resistant isoform (PrP^{Sc}) of prion protein (PrP) is the central event in transmissible spongiform encephalopathies or prion diseases. Differences in transmissibility and susceptibility are largely determined by polymorphisms in PrP, but the exact molecular mechanism behind PrP conversion and the modulation by disease associated polymorphisms is still unclear. To assess whether the polymorphisms in either PrP^{C} or PrP^{Sc} modulate the initial binding of PrP^{C} to PrP^{Sc} we used several naturally occurring allelic variants of sheep PrP^{C} and PrP^{Sc} that are associated with differential scrapie susceptibility and transmissibility (the phylogenetic wild type (ARQ), the codon 136Val variant (VRQ) and the codon 171Arg variant (ARR). Under cell-free PrP conversion conditions known to reproduce the observed in vivo differential scrapie susceptibility we found that relative amounts of PrP^{C} allelic variants bound by various allelic PrP^{Sc} variants are PrP specific and have comparable binding efficiencies. Therefore the differential rate limiting step in conversion of sheep PrP variants is not determined by the initial PrP^{C}-PrP^{Sc} binding efficiency, but seems to be an intrinsic property of PrP^{C} itself. Consequently, a second step after PrP^{C}-PrP^{Sc} binding should determine the observed differences in PrP conversion efficiencies. Further study of this second step may provide a future tool to determine the mechanism underlying refolding of PrP^{C} into PrP^{Sc} and supports the use of conversion-resistant polymorphic PrP^{C} variants as a potential therapeutic approach to interfere with PrP conversion in TSE development.

Introduction.

Transmissible spongiform encephalopathy (TSE) diseases are fatal neurodegenerative disorders and include (among others) familial, sporadic and variant Creutzfeldt-Jacob disease in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. TSEs (or prion diseases) are characterized by formation and accumulation of protease resistant prion protein (PrP^{Sc}) mainly in tissues of the central nervous system. Formation of PrP^{Sc} is a posttranslational process and involves refolding (conversion) of the host-encoded prion protein (PrP^{C}) into partially protease resistant forms (PrP^{Sc}) [1].

Scrapie in small ruminants (e.g. sheep) is one of the best documented models for natural TSE transmission. Polymorphisms in PrP have been shown to be of importance in both interspecies and intraspecies transmissibilities [2]. Susceptibility of sheep to scrapie seems mainly dictated by polymorphisms in the gene coding for the prion protein itself and to date over 20 different, naturally occurring polymorphisms (only one mutation per allele) of PrP have been described [3-11]. The effects of polymorphisms in ovine PrP on the relative susceptibility of sheep to scrapie have been gauged in epidemiological studies of natural scrapie outbreaks, in experimental transmissions to and from sheep, and in cell-free conversion assays [5, 12-16]. Polymorphisms at sheep PrP amino acid position 136, 154 and 171 have been shown most relevant in association with differential TSE susceptibility. Several studies have shown that an alanine at position 136, arginine at position 154 and glutamine at position 171 (ARQ) to be the phylogenetic wild type PrP (wt), with intermediate susceptibility to scrapie. The polymorphism associated with increased susceptibility to scrapie is the substitution alanine with valine at codon 136 (VRQ; 136V) and thus far the only polymorphism shown to be associated with decreased susceptibility or even resistance to natural scrapie is the substitution of glutamine with arginine at codon 171 (ARR; 171R). Cell-free conversion of PrP^{C} provides an excellent *in vitro* model in which relative amounts of produced protinase K (PK) resistant PrP reflect important biological aspects of TSE at the molecular level [2, 14, 16-20]. In sheep scrapie this technique has shown that 136V and wt-PrP^{C} are readily converted in PK resistant PrP by various types of PrP^{Sc} isolated from sheep having different PrP genotypes. In contrast, 171R-PrP is hardly converted into PK resistant PrP [2, 14, 16, 19, 20].

Studies on the conversion of hamster and mouse PrP^{C} isoforms resulted in indications that diminished acquisition of PK resistance is not due to lack of binding of PrP^{C} to PrP^{Sc} [21]. However, no data on binding efficiencies of ovine PrP^{C} to PrP^{Sc} is available to date. Furthermore, whereas differences in susceptibility of- and transmissibility in sheep

can entirely be explained at the molecular level by the effects of single polymorphisms in PrP^C or PrP^{Sc} on PrP conversion, the exact molecular mechanism determining these differences is still unknown [16, 22-24]. In the present study sheep scrapie-susceptibility linked polymorphisms were used to determine whether differential binding efficiencies of sheep PrP^C to PrP^{Sc} determine the observed differential conversion efficiencies of sheep PrP [14, 16, 18, 20].

Results.

Binding efficiencies of PrP^C to PrP^{Sc}.

In total six independent PrP^{Sc} isolates from six sheep homozygous for 136V-PrP^C and six independent PrP^{Sc} isolates from six sheep homozygous for wt PrP^C were isolated and tested for binding affinities to three natural allelic variants of sheep PrP^C; 136V-PrP^C (VRQ), wt-PrP^C (ARQ) and 171R-PrP^C (ARR). At least two independent reaction duplicates were analyzed from each PrP^{Sc} isolate. Binding efficiencies of the individual PrP^{Sc} isolates were determined using the conversion-binding assay in which cell-free conversion conditions were used identical to previous studies showing significant differential conversion of sheep PrP^C variants [16]. Because aggregates were pelleted by spinning 30 minutes at 14.000 rpm, we needed to take into account that not all of the PrP^{Sc} is actually pelleted at this 'low' speed. However, most of the PrP^{Sc} was pelleted (~86.4 %

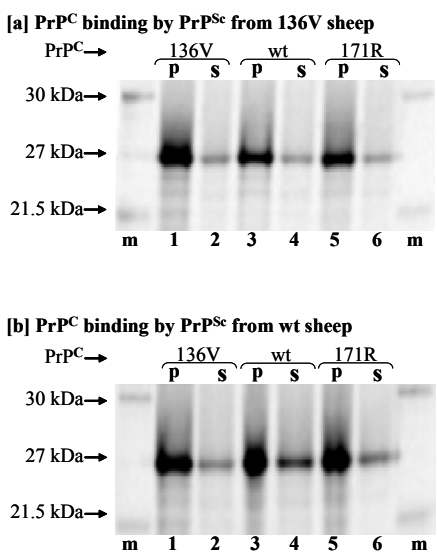

Figure 1. ***Phosphor-image of SDS-PAGE showing an example of binding assay samples obtained with PrP^{Sc} isolated from a sheep homozygous for 136V PrP allele [a] or from a sheep homozygous for wt PrP allele [b].*** *Lanes containing ^{14}C-marker (Amersham biosciences) are marked 'm', sizes of the marker bands are 30 and 21,5 kDa. Lane 1+2 represent results with 136V-PrP^C, lane 3+4 represent results with wt-PrP^C and lane 5+6 represent results with 171R-PrP^C of which the odd lanes contain the pelleted (bound) fraction (p) and the even lanes contain the supernatant (unbound) fraction (s).*

of the total input). Therefore it can be assumed that the amount of ^{35}S-PrP^{C} found in the pellet fraction is representative of most if not all of the actual bound ^{35}S-PrP^{C}. Addition of PrP^{Sc}, isolated from sheep homozygous for 136V-PrP (Fig.1a) or isolated from sheep homozygous for wt-PrP (Fig.1b) resulted in recovering most of the labeled PrP^{C} in the bound pellet [p] fraction (Fig.1a+b, lane 1, 3 and 5) and only a small amount of labeled PrP^{C} remained in the unbound supernatant [s] fraction (Fig.1a+b, lane 2, 4 and 6) for each PrP^{C} tested (table 1; 136V-PrP^{Sc} and wt-PrP^{Sc} isolates). Comparison between repeated measurements of a PrP^{Sc} isolate or between different PrP^{Sc} isolates showed that the absolute amount of bound PrP^{C} was linked to the isolated batch of PrP^{Sc} (probably due to differences in preparation of PrP^{Sc} aliquots (sonication) or 'age' of an isolate). Therefore the absolute binding percentages (per measurement) of bound ^{35}S-PrP^{C} were not compared, but rather the mean binding percentages of repeated measurements (table 1).

Table 1. ***Average percentages of bound ^{35}S-PrP^{C} determined for PrP^{Sc} isolates from sheep.***

			[3]mean bind% PrP^{C}									mean bind%		
isolate	**[1]Type**	**[2]n**	**136V**	**±**	**s.e.m.**	**ARQ**	**±**	**s.e.m.**	**171R**	**±**	**s.e.m.**	**PrP^{Sc} isolate group**	**±**	**s.e.m.**
553237	136V	3	83.3	±	11,6	91.3	±	2,7	89.4	±	2,1			
558998	136V	6	89.2	±	2,8	86.4	±	5,4	89.7	±	5,3			
577275	136V	2	97.0	±	0,5	98.4	±	0,5	89.2	±	2,5	**86.2**	**±**	**1.7**
601936(343)	136V	3	81.9	±	8,5	83.7	±	4,6	91.1	±	1,5			
606316	136V	4	86.3	±	2,2	74.5	±	4,8	84.1	±	4,2			
609619	136V	5	84.0	±	1,1	67.5	±	4,4	85.7	±	0,4			
514076	wt (ARQ)	3	88.1	±	4,7	81.2	±	10,7	88.9	±	9,1			
523900	wt (ARQ)	4	80.1	±	5,3	85.0	±	6,9	70.3	±	9,8			
532643	wt (ARQ)	2	89.2	±	2,3	86.5	±	3,9	74.3	±	19,7	**87.3**	**±**	**1.8**
577331	wt (ARQ)	3	91.0	±	1,6	87.6	±	6,0	85.2	±	4,1			
603397	wt (ARQ)	3	87.9	±	7,2	87.4	±	1,4	93.5	±	1,0			
614116	wt (ARQ)	2	97.9	±	1,4	98.8	±	0,3	98.2	±	0,8			
mean bind%	**PrP^{C} variant**		**88.0**	**±**	**1.6**	**85.6**	**±**	**2.5**	**86.6**	**±**	**2.2**			

[1] PrP^{Sc} isolated from sheep with a homozygous allele for either 136V PrP^{C} or wt PrP^{C}.
[2] the number of repeated (independent) reactions for each PrP^{Sc} isolate.
[3] absolute amounts of bound ^{35}S-PrP^{C} ranged from ~60-99%, depending on the aggregated state of a PrP^{Sc} isolate. Therefore, analysis of variance on the observed binding patterns between PrP^{C} variants and PrP^{Sc} isolate groups was performed.

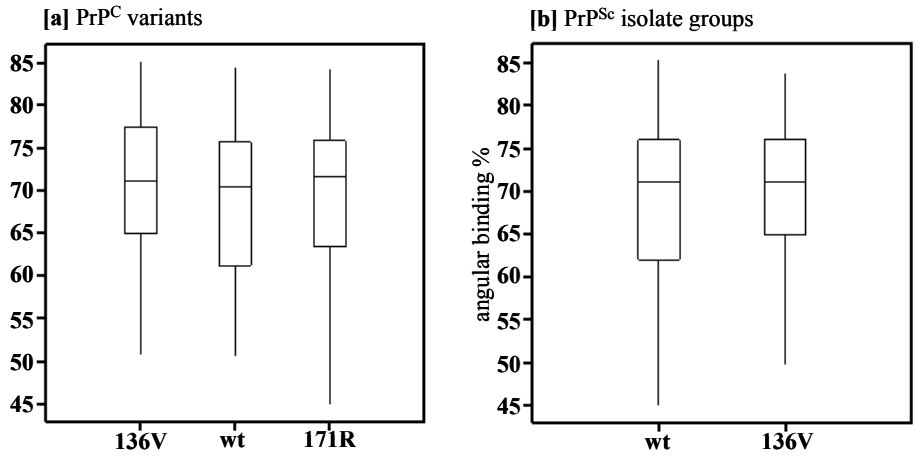

Figure 2. ***Boxplots of binding patterns after angular transformation of binding percentages.*** *The binding percentages have been plotted against the PrP^C variants [a] and PrP^{Sc} isolate groups [b]. The boxplot shows the total spread of all determined binding percentages, with the box representing 95% of all measurements and the line in the box representing the mean value of the measurements. Analysis of variance of the binding percentages shows that neither the PrP^C variant nor the PrP^{Sc} isolate group have a significant effect on the binding patterns obtained.*

Binding percentages were compared by variance analysis, after variance stabilizing angular transformation of the binding percentages. First, binding patterns were compared between the PrP^C variants and no significant differences were found (fig. 2a) since the least significant difference (l.s.d.) between PrP^C variants was calculated to be 4.5% which was higher than the maximum difference of 2.4% between the mean binding percentages of 136V, wt and 171R-PrP^C (88.0±1.6%, 85.6±2.5% and 86.6±2.2% respectively). Secondly, binding patterns were compared between the PrP^{Sc} groups and again no significant differences were shown (fig. 2b) since the l.s.d. between PrP^{Sc} variants was 5.8% which is again higher than the maximum difference of 1.1% between the mean binding percentages for the wt/wt (homozygous wt-PrP) and 136V/136V (homozygous 136V-PrP) PrP^{Sc} isolate groups (86.2±1.7% and 87.3±1.8% respectively).

To gain insight in the dynamics of the binding reaction, binding percentages were also determined at shorter incubations (one hour, one day, two days, and the standard three days). At each time point binding percentages were determined of the three PrP^C variants to PrP^{Sc} (n=4; 2x wt/wt + 2x 136V/136V). No significant differences were found between the three PrP^C variants and the overall average binding percentages at the four time points (fig. 3).

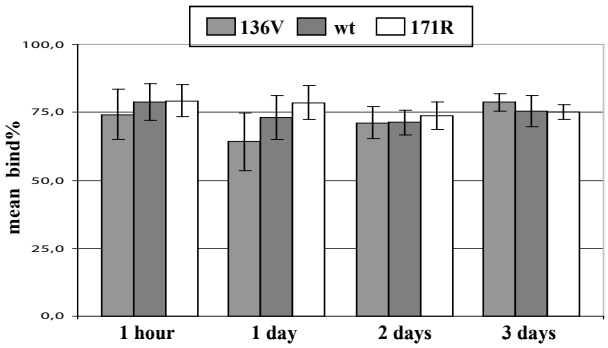

Figure 3. ***Kinetics of the binding efficiencies of the three PrP^{C} variants to PrP^{Sc} homozygous 136V and homozygous wt.*** *Mean binding percentages for each PrP^{C} variant are indicated including the error bars (s.e.m.) for the repeated experiments after incubation for the indicated time.*

Binding specificity of PrP^{Sc} to PrP^{C}.

In order to exclude potential aspecific binding or aggregation features of PrP^{C}, we performed several control. First of all ^{35}S-PrP^{C} was incubated without addition of PrP^{Sc} to determine whether 'self aggregation' and spontaneous pelleting of PrP^{C} occurred. Exclusion of PrP^{Sc} by replacing with demineralized water (SQ) did not result in significant amounts of ^{35}S-PrP^{C} in the pellet fraction leaving on average 93.6±1.3% of the ^{35}S-Prp^{C} variants in the supernatant fraction (Fig 4a lane 1+2). Therefore self-aggregation of PrP^{C} is not responsible for recovering significant amounts of labeled PrP^{C} in the pellet. Furthermore it shows that the presence of aggregated protein (PrP^{Sc}) is a prerequisite for pelleting ^{35}S-PrP^{C} under conditions maintaining cell-free conversion specificity.

To determine whether ^{35}S-PrP^{C} could be aspecifically 'captured' and pelleted by any aggregated protein, PrP^{Sc} was replaced in the binding assay by keyhole limpet hemocyanin (KLH), a very large mainly aggregated protein. A surplus of KLH was added (about 5 µg per reaction) to favor KLH aggregation. No significant amounts of ^{35}S-PrP^{C} were detected in the pellet fraction. On average 94.3±2.3% of the ^{35}S-PrP^{C} remained in the unbound fraction (Fig.4a, lane 3+4), which is about the same as the amount of PrP^{C} in the pellet fraction of assays without any aggregated protein (paragraph above). To ensure that KLH remained aggregated under the used specific conversion conditions, soluble and pellet fractions were also analyzed on SDS-PAGE by total protein staining (Sypro Orange, Molecular Probes). On average 79.8±1.0% of the added KLH was recovered in the pellet fraction (example; Fig.4b, lane 1+2), which is comparable to the percentage of aggregation determined for KLH (75.9 ± 2.1%) in storing buffer (0.1M Na-phosphate

buffer, pH 7). Since no increase in PrP^C pelleting was observed, and although only one other aggregated protein was tested for aspecific capture of PrP^C, the ^{35}S-PrP^C is probably not a "sticky" protein binding to any aggregate.

To determine whether ^{35}S-PrP^C could aspecifically bind to other large soluble proteins, resulting in significant amounts of precipitation, PrP^{Sc} was replaced by thyroglobulin (TG) a large unrelated soluble protein frequently used as carrier in protein precipitation methodologies. A surplus of TG was added (about 5 µg per reaction) but no significant amounts of ^{35}S-PrP^C were detected in the pellet fraction; on average 90.6±2.6% remained in the supernatant fraction (Fig.4a, lane 5+6). To ensure that TG remained largely soluble under the used specific conversion conditions, soluble and pellet fractions were also analyzed on SDS-PAGE by total protein staining (Sypro Orange). On average 94.4±2.5% of the TG remained in the supernatant (Fig.4b, lane 3+4) under the used specific conversion conditions. Even though only one large soluble protein was tested, this indicates that ^{35}S-PrP^C is not significantly precipitated by binding to any other large soluble heterologous protein like TG.

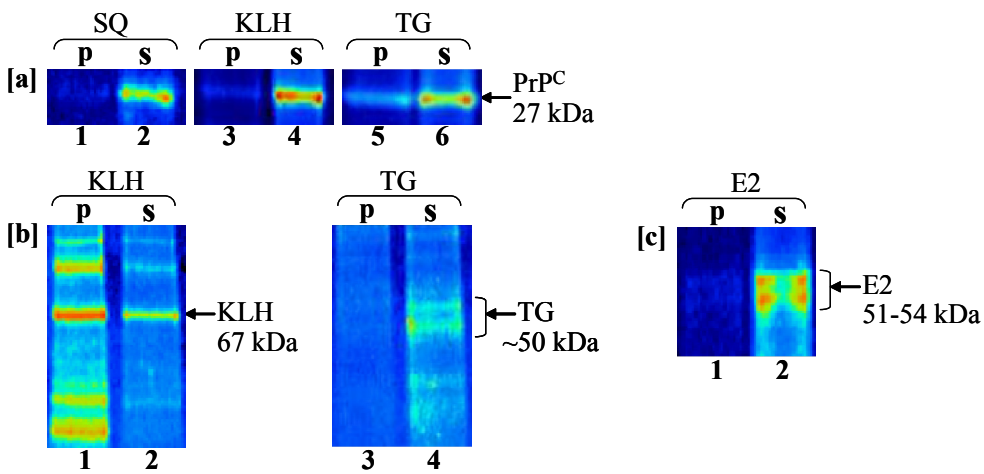

Figure 4. ***Examples of control reactions for determining specificity of PrP^C – PrP^{Sc} interaction within the binding assay.*** *[a] Phosphor-image of SDS-PAGE (indicated in pseudo intensity staining) analysis of samples obtained when PrP^{Sc} was replaced with either water (SQ, lane 1+2), KLH (lane 3+4) or TG (lane 5+6). [b] Sypro Orange total protein staining of SDS-PAGE (indicated in pseudo intensity staining) containing conversion/binding samples having KLH (lane 1+2) and TG (lane 3+4) instead of PrP^{Sc} under the used specific conversion conditions. [c] Phosphor-image of SDS-PAGE (indicated in pseudo intensity staining) containing samples obtained when ^{35}S-PrP^C was replaced by ^{35}S-E2 protein of CSFV. All odd lanes contain the pelleted (bound) fraction (p) and all even lanes contain the supernatant (unbound) fraction (s).*

To determine whether any labeled soluble protein would bind to the added aggregated PrP^{Sc}, ^{35}S-PrP^{C} was replaced with ^{35}S-E2 of CSFV, an unrelated but similarly processed protein (membrane bound partially N-glycosylated protein of about 51-54 kDa). No significant amounts of labeled ^{35}S-E2 protein were found in the bound fraction, while 91.9±3.5% of the ^{35}S-E2 protein remained in the unbound fraction (Fig.4c, lane 1+2). This indicates that binding of ^{35}S-PrP^{C} by PrP^{Sc} is PrP specific.

In summary, we have shown that PrP^{C} binds efficiently to PrP^{Sc} with no significant differences in binding patterns between PrP^{C} variants and PrP^{Sc} isolate groups, under conditions maintaining cell-free conversion specificity (Fig.5). Furthermore we have shown that PrP^{C} does not spontaneously aggregate due to the specific conversion condition used, does not stick to unrelated aggregated protein like KLH and does not precipitate with other large soluble proteins like TG. Additionally we have shown that PrP^{Sc} does not bind to unrelated labeled soluble protein (E2) as well. Therefore we can conclude that PrP^{Sc} associated pelleting of ^{35}S-PrP^{C} represents a PrP^{C}–PrP^{Sc} specific interaction and that addition of aggregated protein (PrP^{Sc}) is a prerequisite for pelleting ^{35}S-PrP^{C} under conditions maintaining cell-free conversion specificity. Since no differences were detected in the binding patterns of the tested PrP^{C} variants to the different PrP^{Sc} isolates, the rate limiting step determining the observed differential conversion efficiencies of PrP^{C} variants has to be during a subsequent step in the conversion after binding of PrP^{C} to PrP^{Sc} (Fig.6).

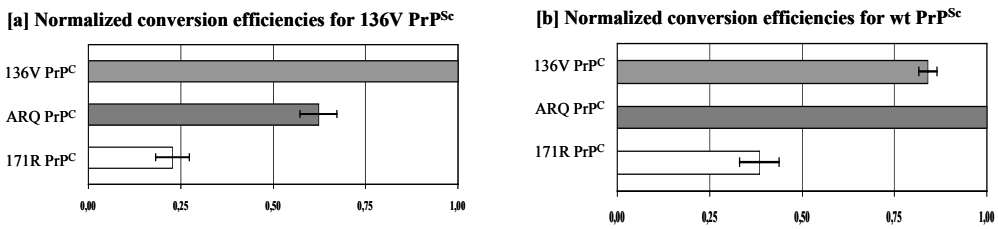

Figure 5. ***Observed relative normalized conversion efficiencies of the combined conversion binding reactions.*** *Reactions were normalized to the most efficient homologous reactions. Panel a contains the conversion efficiencies of the three PrP^{C} variants induced by 136V-PrP^{Sc}. Panel b contains the reactions induced by wt-PrP^{Sc}. Mean normalized efficiencies and the corresponding error bars (s.e.m.) are indicated.*

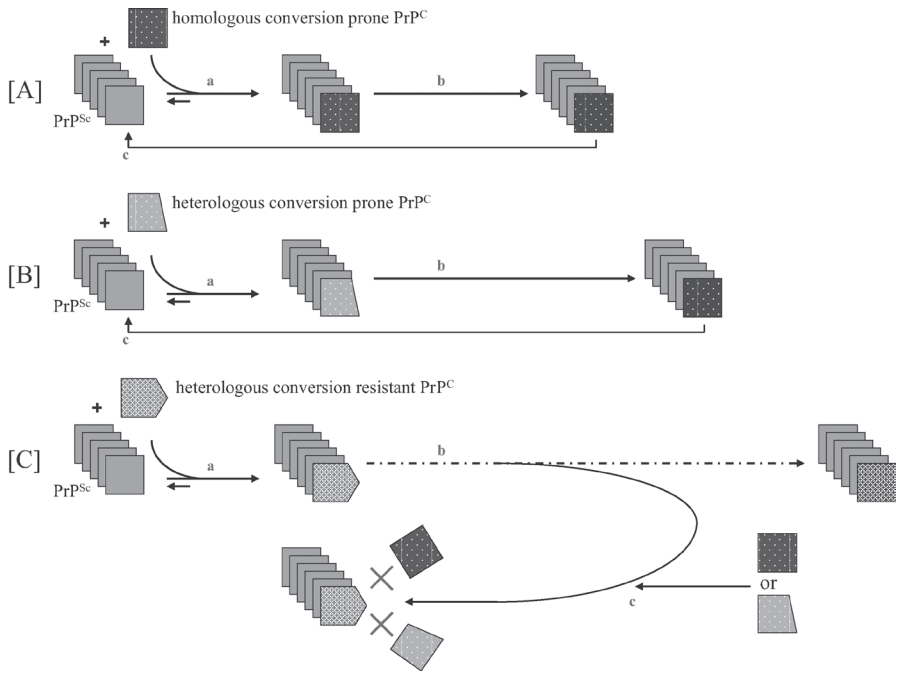

Figure 6. ***Schematic representation of PrP^C conversion.*** *[A] Conversion of homologous, conversion prone PrP^C (wt, 136V): efficient binding (a), highly efficient conversion (b) and 'reseeding' (c) possible.* ***[B]*** *Conversion of heterologous, conversion prone PrP^C (wt, 136V): efficient binding (a), efficient conversion (b) and 'reseeding' (c) possible.* ***[C]*** *Conversion of heterologous, conversion resistant PrP^C (171R): efficient binding (a), highly inefficient or blocked conversion (b) and 'reseeding' (c) possibly blocked.*

Discussion.

The aim of this study was to gain insight into the mechanism underlying the modulation of sheep scrapie susceptibility by polymorphisms in PrP^C or PrP^{Sc}. Bossers *et al.* (2000) showed that the *in vitro* conversion assay is a representative tool for assessing modulating effects of scrapie-associated polymorphisms. Other studies have shown that the conversion of PrP^C by PrP^{Sc} is induced by the aggregated forms of PrP^{Sc} [17, 25]. These aggregates can be pelleted by high-speed centrifugation. The amount of ^{35}S-PrP^C that is bound by PrP^{Sc} and subsequently recovered from the pellet should give an indication whether disease associated polymorphisms modulate binding of PrP^C by PrP^{Sc} or whether these polymorphisms have their modulating effects in a subsequent step after the initial binding during the conversion.

By applying a conversion-binding assay [21], binding efficiencies of sheep PrP^{C} variants to sheep PrP^{Sc} variants could be measured. Since no significant differences in binding efficiencies were measured between any of the variants, the initial binding efficiencies cannot account for the observed differential conversion efficiencies of sheep scrapie susceptibility linked variants of PrP. We show for instance that 171R-PrP^{C} binds to PrP^{Sc} as efficient as 136V-PrP^{C} or wt-PrP^{C}, whereas conversion efficiencies differ remarkably. Therefore a second step (or more) in the conversion process, in which the disease associated polymorphisms have their modulating effect, should be involved in conversion of the PrP protein (Fig.4 a+b). These findings are corroborated by a study in which interactions between heterologous forms of prion protein have been studied *in vitro* using mouse and hamster PrP isoforms [21] in which is shown that PrP^{C} of different species (hamster and mouse) bind equally efficient to PrP^{Sc} of mouse while preserving conversion specificity also indicating that a second step in the conversion after initial binding should determine the species specificity.

In this study we also show that PrP^{C} does not precipitate spontaneously, does not stick to unrelated aggregated protein (KLH), and does not precipitate with other large soluble proteins (TG). In addition we showed that PrP^{Sc} does not bind unrelated labeled soluble protein E2 as well. Therefore we concluded that PrP^{Sc} associated pelleting of ^{35}S-PrP^{C} represents a PrP^{C}–PrP^{Sc} specific interaction and that addition of aggregated PrP^{Sc} is a prerequisite for pelleting ^{35}S-PrP^{C} under conditions maintaining cell-free conversion specificity. These results are in conjunction with results in which no spontaneous PK resistant PrP was formed under the cell-free conversion conditions when PrP^{Sc} was replaced by SQ [14, 16]. The inability of PrP^{Sc} to significantly bind ^{35}S-E2 additionally confirms that binding of ^{35}S-PrP^{C} to PrP^{Sc} is PrP specific and does not solely depend on posttranslational modifications or aspecific "sticky" properties of PrP.

Since 171R-PrP^{C} seems to bind as efficient to PrP^{Sc} as wild-type and 136V-PrP^{C}, the 171R-PrP^{C} variant may be a valuable in; firstly, providing clues for designing new therapeutic strategies by determination of the mechanism underlying the refolding process of PrP^{C} into PrP^{Sc}. For example by using the 171R-PrP^{C} variant to determine sites involved in binding and/or conversion or by comparing protein properties (i.e. stability, unfolding/refolding kinetics). Secondly, since conversion resistant 171R-PrP^{C} binds efficiently to PrP^{Sc} it may provide a future tool to block prion conversion through direct interference or blocking of PrP^{Sc} polymer growth as hypothesized before by Bossers *et al* 1999. In addition, results from literature show that heterozygosity for PrP is a protective factor against TSE development as demonstrated by studies *in vitro*: [21, 26, 27], or in vivo for sheep: [5, 12, 13, 28, 29] and humans: [30, 31]. This is in conjunction with our results

showing that various differentially converting PrP^{C} variants bind equally efficiently to PrP^{Sc} but have different conversion efficiencies. In heterozygotes this "inhibition" of conversion by heterologous PrP variants might explain why heterozygotes have longer incubation times than their homozygous counterparts. This is corroborated by the fact that resistance in heterozygous sheep is not caused by preferential allelic use [32].

The coupled *in vitro* cell-free conversion efficiencies (Fig.5) reflect results as described before [16] where susceptibility to scrapie was linked to the modulating effects of polymorphisms on the conversion of sheep PrP. In addition to these *in vitro* cell-free conversion assays, PrP polymorphisms have been shown to tightly control sheep prion replication in cultured cells as well [24]. Furthermore, it has been shown that polymorphisms in PrP determine both interspecies and intraspecies transmissibilities [2, 19] and/or the stability of the PrP^{C} molecule itself [33].

By correlating conversion- and binding patterns we showed that 171R-PrP^{C} binds to PrP^{Sc} as efficiently as conversion prone variants like wt (ARQ) and 136V-PrP^{C}. Since naturally occurring polymorphisms of sheep PrP^{C} seem not to have a significant modulating effect on the initial binding of PrP^{C} to PrP^{Sc}, these could somehow modulate a subsequent step in the conversion process (Fig.6 a+b). Both 136V and 171R are polymorphisms that affect PrP^{C} stability and are close to the region that supposedly is involved in refolding of PrP^{C} to PrP^{Sc} [33, 34]. This region is comprised of the two small β-sheets (sheep amino acid residues 129-134 [S1] and 163-167 [S2]) which are the positions from where the first α-helix (amino acid residues 146-158 [H1]) is converted into an anti-parallel organized β-sheeted structure. It could also be that the 171R polymorphism results in increased protease sensitivity of 171R PrP^{C} itself due to destabilization of the PrP^{C} molecule [33] and thus resulting in slower amyloidogenesis, because the 171R-PrP^{C} molecule could internalize and degrade by the PrP expressing cell more rapidly than the other variants before the actual polymerization can take place. In contrast, the 136V polymorphism could stabilize the PrP^{C} molecule, resulting in an elongating of the survival of 136V-PrP^{C} and thereby supporting the subsequent conversion.

Since disease associated polymorphisms of sheep PrP do not have an effect on binding properties of PrP^{C} to PrP^{Sc}, dominant negative inhibition of the 171R polymorphism on prion conversion [15, 16, 19, 35] is therefore not due to lack of interaction between PrP variants as suggested by Perrier 2002, but more probably due to a more subtle mode of modulation by the PrP polymorphism on the conversion or on the interaction with chaperone proteins under natural conditions.

This study shows that the interaction between PrP^{C} – PrP^{Sc} in de conversion-binding assay is PrP specific. Whether PrP^{C} binds to the so-called nucleation site of PrP^{Sc} only or whether it can bind to other sites of PrP^{Sc} aggregates is under investigation. The next logical step currently under investigation is to find out whether conversion resistant (natural or artificial) PrP variants can effectively interfere with the process of PrP conversion and thereby therapeutically block or significantly delay TSE development.

Experimental procedures.

PrP^{C} constructs and expression.

The three sheep PrP^{C} variants used (136V, wt and 171R) were cloned, expressed and characterized as described before [14]. Briefly; PrP open reading frames were subcloned in the eukaryotic expression vector pECV7. The vectors containing PrP open reading frames were stably transfected into CHO cells. High and stable expressing single-cell-clones were selected by immunoperoxidase monolayer assay and Western blotting, using rabbit anti- peptide antiserum R521-7 [36].

Radio-labelling and purification of PrP^{C}.

Radio-labelling and purification of the three PrP^{C} variants was performed as described before [16, 18]. Briefly; single cells clones expressing the different PrP^{C} variants were starved for 30-60 minutes in label medium and subsequently labeled with 1mCi [^{35}S]-methionine/cysteine TRAN^{35}S-label (ICN Biomedicals Inc.). Cells were lysed in Triton X-100 (0.5%, ICN Biomedicals Inc.) containing lysisbuffer in the presence of protease inhibitors (1 nM Pefabloc SC, 1 nM leupeptine, 1 nM pepstatin and 0.15 nM aproprotin). ^{35}S-PrP^{C} was immunopurified by PrP specific antiserum R521-7 captured by Protein A Sepharose (10% w/v), which was eluted in 0.1M acetic acid.

Radiolabelling and purification of Classical Swine Fever Virus glycoprotein E2.

For labeling an expression-vector containing the gene coding for glycoprotein E2 of classical swine fever virus (CSFV) transfected in sk6 cells [37] was used. Radio-labeling and purification of E2 was essentially performed as described above albeit on a larger scale. ^{35}S-E2 was immunopurified using monoclonal antibody V3 [38], and also eluted in 0.1M acetic acid.

PrP^{Sc} purification and analysis.

PrP^{Sc} was isolated from brain tissue of clinically ill scrapie sheep with either homozygous alleles for 136V PrP or wt PrP. PrP genotypes were determined by Sanger sequencing of the full PrP-ORF as described before [5]. PrP^{Sc} was purified by ultracentrifugational pelleting from sarcosyl-homogenated brains as described previously [14, 17]. The final pellets were sonicated in phosphate-buffered saline containing 1.0% zwitter-reagent (SB 3-14). Yields of PrP^{Sc} were quantified by SDS-PAGE (12% NuPAGE, Invitrogen) and Western blotting using antiserum R521-7.

Conversion-Binding assay.

Conversion and binding efficiencies were determined by double volume cell-free conversion reactions essentially as described [21, 39] adapted to cell-free conversion conditions as used before [14, 16, 17]. Briefly; PrP^{Sc} was partially denatured in 2.5M Guanidin-hydrochloride (GdnHCl) for at least 2.5 hours at 37°C. Aliquots of denatured PrP^{Sc} (2-4 μg per reaction) were mixed with 10,000-20,000cpm purified ^{35}S-PrP^{C} (~20-40ng ^{35}S-PrP^{C}) and further diluted to a final concentration of 1.0M GdnHCl in conversion buffer (50mM sodium-citrate, pH 6.0, 5mM cetylpyridinium chloride, 1% N-laurylsarcosine and protease inhibitors). Reactions were incubated for 3 days at 37°C (or shorter for the kinetic experiments). After incubation the reaction volume was split in two equal aliquots in separate siliconized tubes. One aliquot was used for binding analysis and centrifuged for 30 minutes at 14.000 rpm at room temperature, the supernatant transferred to a separate siliconized tube (unbound fraction) and the pellet (bound fraction) dissolved in 1% SDS by sonication. From the second aliquot $^{1}/_{10}$ volume was transferred to a separate siliconized tube (reference fraction) and the remaining $^{9}/_{10}$ volume was treated with 35 μg/ml protienase K (PK) for 1 hour at 37°C. PK was inactivated by addition of Pefabloc-SC (Roche).

All the samples were methanol precipitated and the pellet was dried and dissolved in Leammli SDS-PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol and 4M urea. Samples were run on SDS-PAGE (12% NuPAGE, Invitrogen), the dried gels were visualized by phosphorimaging and analyzed using a STORM-840 imager and the ImageQuant 5.1 software (Molecular Dynamics). Binding percentages were calculated by dividing the amount of labeled PrP^{C} (MW between 24 and 28.5 kDa) of each fraction (pellet [p] and supernatant [s]) by the total amount of labeled PrP^{C} (p+s). Conversion percentages were calculated by dividing the amount of labelled PrP left after PK digestion (MW between 19 and 21.5 kDa) by the amount of labeled PrP^{C} in the reference fraction (MW between 24 and 28.5 kDa).

Statistical Analysis of binding efficiencies.

Statistical calculations were performed using the GenStat 6.1 program. To compensate for differences in possible variance as a result of a fixed scale, variance stabilizing angular transformation of the binding percentages was utilized (section 4.1.3 [40]). Absolute amounts of bound ^{35}S-PrP^{C} varied probably as a result of the aggregated state of the PrP^{Sc} isolate used; therefore binding patterns were compared by the analysis of variance method to determine whether significant differences occurred in these binding patterns. Comparisons of binding patterns were made separately for the three PrP^{C} variants and for the two different PrP^{Sc} isolate groups. In order to determine significant differences in binding patterns, the least significant difference (l.s.d.; the minimum required amount needed to demonstrate a significant difference) was calculated and compared to the observed differences between the mean binding percentages for either the PrP^{C} variants or the PrP^{Sc} isolate groups.

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References.

1. DeArmond, S.J. and S.B. Prusiner, *Perspectives on prion biology, prion disease pathogenesis, and pharmacologic approaches to treatment.* Clin Lab Med, 2003. 23(1): p. 1-41.
2. Bossers, A., et al., *In vitro conversion of normal prion protein into pathologic isoforms, in Clin Lab Med,* B. Ghetti, MD and P. Piccardo, MD, Editors. 2003, W.B. Saunders Company, a division of Elsivier Science. p. 227-47.
3. Goldmann, W., et al., *Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene*. J Gen Virol, 1991. 72 (Pt 10): p. 2411-7.
4. Goldmann, W., et al., *Two alleles of a neural protein gene linked to scrapie in sheep.* Proc Natl Acad Sci U S A, 1990. 87(7): p. 2476-80.
5. Bossers, A., et al., *PrP genotype contributes to determining survival times of sheep with natural scrapie.* J Gen Virol, 1996. 77 (Pt 10): p. 2669-73.
6. Belt, P.B.G.M., et al., *PrP allelic variants associated with natural scrapie., in Bovine Spongiform Encephalopathy; The BSE dilemma,* C.J. Gibbs Jr., Editor. 1996, Springer: New York. p. 294-305.
7. Junghans, F., et al., *Genotyping of German sheep with respect to scrapie susceptibility.* Vet Rec, 1998. 143(12): p. 340-1.
8. Elsen, J.M., et al., *Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov.* Arch Virol, 1999. 144(3): p. 431-45.
9. Thorgeirsdottir, S., et al., *PrP gene polymorphism and natural scrapie in Icelandic sheep.* J Gen Virol, 1999. 80 (Pt 9): p. 2527-34.
10. Tranulis, M.A., et al., *Prion protein gene polymorphisms in sheep with natural scrapie and healthy controls in Norway.* J Gen Virol, 1999. 80 (Pt 4): p. 1073-7.
11. O'Rourke, K.I., et al., *Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue.* J Clin Microbiol, 2000. 38(9): p. 3254-9.
12. Goldmann, W., et al., *PrP genotypes and the Sip gene in Cheviot sheep form the basis for scrapie strain typing in sheep.* Ann N Y Acad Sci, 1994. 724: p. 296-9.
13. Hunter, N., et al., *Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes.* Arch Virol, 1996. 141(5): p. 809-24.
14. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.
15. Bossers, A., F.L. Harders, and M.A. Smits, *PrP genotype frequencies of the most dominant sheep breed in a country free from scrapie.* Arch Virol, 1999. 144(4): p. 829-34.

16. Bossers, A., R. de Vries, and M.A. Smits, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol, 2000. 74(3): p. 1407-14.
17. Caughey, B., et al., *Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state.* Chem Biol, 1995. 2(12): p. 807-17.
18. Raymond, G.J., et al., *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans.* Nature, 1997. 388(6639): p. 285-8.
19. Bossers, A., *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie.* 1999, University of Utrecht.
20. Raymond, G.J., et al., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo J, 2000. 19(17): p. 4425-30.
21. Horiuchi, M., et al., *Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers.* Proc Natl Acad Sci U S A, 2000. 97(11): p. 5836-41.
22. Dubois, M.A., et al., *Multiplicative genetic effects in scrapie disease susceptibility.* C R Biol, 2002. 325(5): p. 565-70.
23. Tranulis, M.A., *Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep.* Apmis, 2002. 110(1): p. 33-43.
24. Sabuncu, E., et al., *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol, 2003. 77(4): p. 2696-700.
25. Caughey, B., et al., *Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies.* J Virol, 1997. 71(5): p. 4107-10.
26. Priola, S.A., et al., *Heterologous PrP molecules interfere with accumulation of protease-resistant PrP in scrapie-infected murine neuroblastoma cells.* J Virol, 1994. 68(8): p. 4873-8.
27. Holscher, C., H. Delius, and A. Burkle, *Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation.* J Virol, 1998. 72(2): p. 1153-9.
28. Belt, P.B., et al., *Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie.* J Gen Virol, 1995. 76 (Pt 3): p. 509-17.
29. Clouscard, C., et al., *Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie.* J Gen Virol, 1995. 76 (Pt 8): p. 2097-101.
30. Collinge, J., M.S. Palmer, and A.J. Dryden, *Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease.* Lancet, 1991. 337(8755): p. 1441-2.
31. Palmer, M.S., et al., *Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease.* Nature, 1991. 352(6333): p. 340-2.

32. Caplazi, P.A., K.I. O'Rourke, and T.V. Baszler, *Resistance to scrapie in PrP ARR/ARQ heterozygous sheep is not caused by preferential allelic use.* J Clin Pathol, 2004. 57(6): p. 647-50.

33. Rezaei, H., et al., *Amyloidogenic unfolding intermediates differentiate sheep prion protein variants.* J Mol Biol, 2002. 322(4): p. 799-814.

34. Eghiaian, F., et al., *Insight into the PrPC-->PrPSc conversion from the structures of antibody-bound ovine prion scrapie-susceptibility variants.* Proc Natl Acad Sci U S A, 2004. 101(28): p. 10254-9.

35. Perrier, V., et al., *Dominant-negative inhibition of prion replication in transgenic mice.* Proc Natl Acad Sci U S A, 2002. 99(20): p. 13079-84.

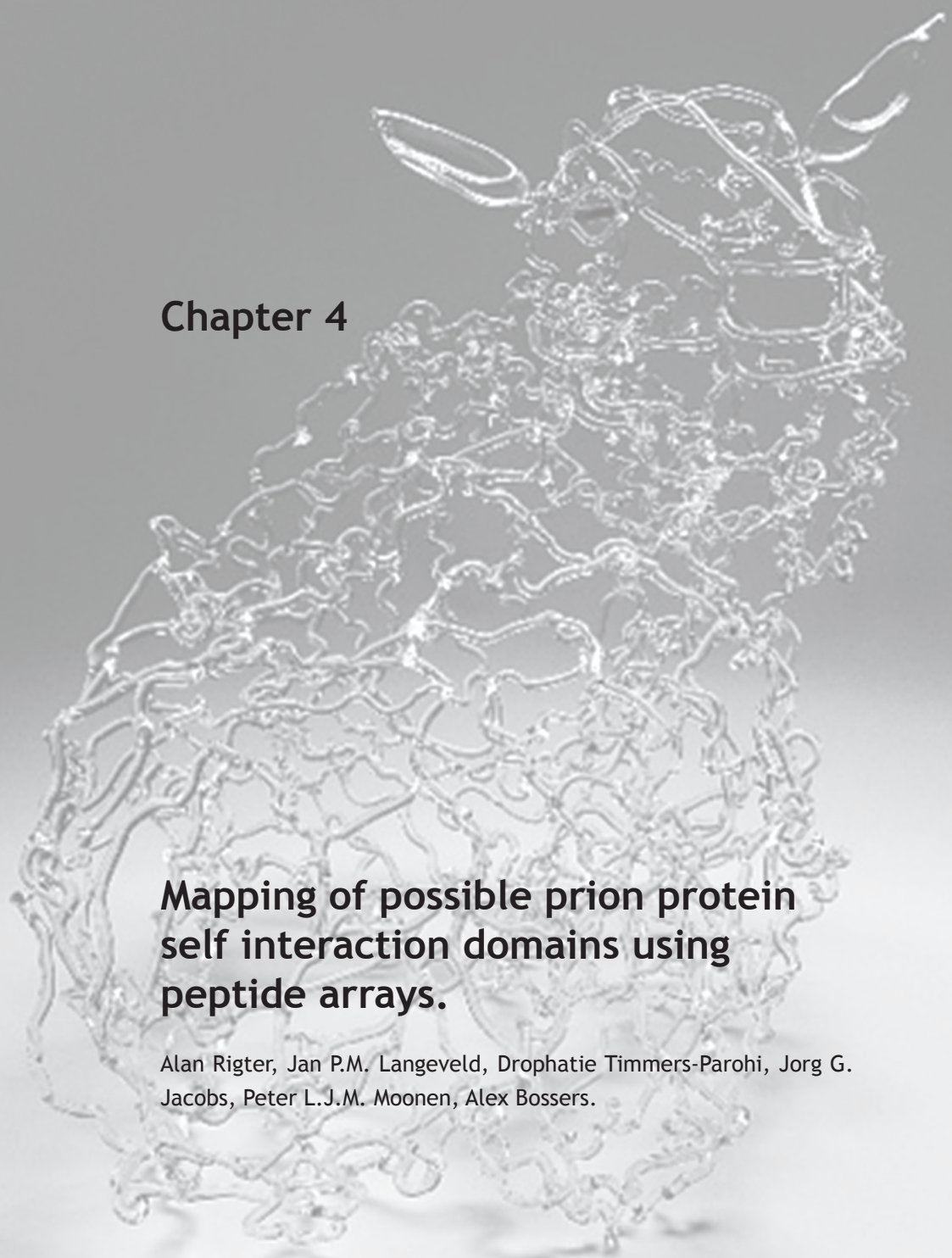
36. van Keulen, L.J., et al., *Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie.* Vet Pathol, *1995. 32(3): p. 299-308.*

37. van Gennip, H.G., et al., Experimental non-transmissible marker vaccines for classical swine fever (CSF) by trans-complementation of E(rns) or E2 of CSFV. Vaccine, 2002. 20(11-12): p. 1544-56.

38. Wensvoort, G., *Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies.* J Gen Virol, 1989. 70 (Pt 11): p. 2865-76.

39. Priola, S.A. and V.A. Lawson, *Glycosylation influences cross-species formation of protease-resistant prion protein.* Embo J, 2001. 20(23): p. 6692-9.

40. McCullagh, P.N., J A, *Generalized linear models / P. McCullagh, J.A. Nelder. first ed. Monographs on Statistics and Applied Probability.* 1983: London, England, Chapman and Hall. 261.



Chapter 4

Mapping of possible prion protein self interaction domains using peptide arrays.

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Mapping of possible prion protein self-interaction domains using peptide arrays.

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Abstract.

Background. The common event in transmissible spongiform encephalopathies (TSEs) or prion diseases is the conversion of host-encoded protease sensitive cellular prion protein (PrP^{C}) into strain dependent isoforms of scrapie associated protease resistant isoform (PrP^{Sc}) of prion protein (PrP). These processes are determined by similarities as well as strain dependent variations in the PrP structure. Selective self-interaction between PrP molecules is the most probable basis for initiation of these processes, potentially influenced by chaperone molecules, however the mechanisms behind these processes are far from understood. We previously determined that polymorphisms do not affect initial PrP^{C} to PrP^{Sc} binding but rather modulate a subsequent step in the conversion process. Determining possible sites of self-interaction could elucidate which amino acid(s) or amino acid sequences contribute to binding and further conversion into other isoforms. To this end, ovine -and bovine PrP peptide-arrays consisting of 15-mer overlapping peptides were probed with recombinant sheep PrP^{C} fused to maltose binding protein (MBP-PrP).

Results. The peptide-arrays revealed two distinct high binding areas as well as some regions of lower affinity in PrP^{C} resulting in total in 7 distinct amino acid sequences (AAs). The first high binding area comprises sheep-PrP peptides 43-102 (AA 43-116), including the N-terminal octarepeats. The second high binding area of sheep-PrP peptides 134-177 (AA 134-191), encompasses most of the scrapie susceptibility-associated polymorphisms in sheep. This concurs with previous studies showing that scrapie associated-polymorphisms do not modulate the initial binding of PrP^{C} to PrP^{Sc}. Comparison of ovine –and bovine peptide-array binding patterns revealed that amino acid specific differences can influence the MBP-PrP binding pattern. PrP-specific antibodies were capable to completely block interaction between the peptide-array and MBP-PrP. MBP-PrP was also capable to specifically bind to PrP in a Western blot approach. The octarepeat region of PrP seems primarily important for this interaction because proteinase K pre-treatment of PrP^{Sc} completely abolished binding.

Conclusions. Binding of MBP-PrP to PrP-specific sequences indicate that several specific self-interactions between individual PrP molecules can occur and suggest that an array of interactions between PrP^{C}-PrP^{C} as well as PrP^{C}-PrP^{Sc} may be possible, which ultimately lead to variations in species barrier and strain differences.

Background.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders characterized by formation and accumulation of partially protease resistant prion protein (PrP^{Sc}) mainly in tissues of the central nervous system. TSEs (or prion diseases) include (among others) familial, sporadic and variant Creutzfeldt-Jacob disease in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep. Formation of PrP^{Sc} is a posttranslational process and involves refolding (conversion) of the host-encoded prion protein (PrP^{C}) into partially protease resistant forms (PrP^{Sc}) [1]. Since no other proteins are known to be involved in this conversion, the existence of a specific and probably efficient self interaction between PrP molecules must be considered.

The molecular mechanism involved in PrP conversion is not well understood, but polymorphisms in PrP have been shown to be of importance in both interspecies and intraspecies transmissibilities [2] and cell-free conversion of PrP^{C} provides an excellent *in vitro* model in which relative amounts of produced proteinase K (PK) resistant PrP reflect important biological aspects of TSEs at the molecular level [2-9]. Whereas differences in susceptibility of- and transmissibility in sheep can largely be explained at the molecular level by the effects of single polymorphisms in PrP^{C} or PrP^{Sc} on PrP conversion [6, 10-12], the exact molecular mechanism of disease development modulation by polymorphisms is still unknown, however we previously showed that disease associated polymorphisms do not affect the initial binding of PrP^{C} to PrP^{Sc} [13]. Hölscher *et al* showed by deletion of residues 114-121 (mouse PrP) the necessity of the highly amyloidogenic AGAAAAGA motif in conversion of PrP^{C} to PrP^{Sc} [14]. Many other studies have revealed the importance of the PrP regions encompassing amino acid sequence (AA) 90-120 (which confirms the importance of AGAAAAGA) [15-17] and 132-156 [8, 15, 18-27]. However, to our knowledge no attempts have been made to systematically map all possible AA involved in PrP interaction (During review of this manuscript a study with complementary results directed at the identification of regions of PrP^{C} that tightly bind to PrP^{Sc} by using a limited set of sequential 24-mer polypeptides motif grafted onto an antibody was published [28]. Our study has its focus however, on systematical domain mapping at the single amino acid level by using a complete set of overlapping 15-mer PrP derived peptides). In order to elucidate which AA of PrP capable of interaction are involved in the primary interaction of PrP^{C} to PrP^{Sc}, a peptide-array based on linear PrP sequences comprising the complete PrP sequence was utilized to determine which residues of PrP are capable of interacting with PrP^{C}.

Results.

MBP-PrP expression and analysis.

Expression of maltose binding protein N-terminally fused to PrP (MBP-PrP) revealed a mainly soluble recombinant MBP-PrP of approximately 70 kDa (Fig.1, lanes 1 & 2) and is readily detected in Western blot using a PrP-specific antibody (9A2, Fig.1B) or a MBP specific antibody (Fig.1C). The MBP-PrP fusion-protein could be purified using the amylase-resin column and the naked PrP protein could be obtained by digestion with protease Factor Xa, indicating accessible folding (Fig.1, lanes 3-6). After 24 hours approximately 45% of MBP-PrP was digested by factor Xa, however when aided by addition of 0.01% SDS factor Xa completely digested MBP-PrP within 24 hours (data not shown). Monoclonal (9A2 and 94B4, Table 1) and polyclonal (R521, epitope AA100-102 [29, 30]) PrP-specific antibodies (Table 1) with specificity for epitopes dispersed throughout the PrP-protein detected MBP-PrP in Western blot.

Table 1. ***Monoclonal antibody overview.***

antibody	epitope[a]	position[b]	blocking[c] (µg/ml)	reference[d]
100B3	KRPKP	26-30	~ 0,5	Thuring, 2005 [30]
SAF32	QPHGGGW [e]	54-92	25,0	Feraudet, 2005 [51]
9A2	WNK	102-104	4,0	Langeveld, 2006 [47]
6C2	HVAGAAA	114-120	20,0	this paper
6H4	DYEDRYYRE	147-155	1,0	Korth, 1997 [18]
94B4	HTVTTTTK	190-197	10,0	Thuring, 2004 [52]
M7	QQSYGQEP	n.a.	n.a.	Bakker, pers. comm.

[a] *amino acid sequence of the reported antibody epitope using peptide mapping techniques*

[b] *position of the antibody epitope(s) in the ovine protein sequence*

[c] *concentration at which the antibody completely blocked binding on the peptide-array*

[d] *publication in which the epitope mapping results are reported*

[e] *epitope of octarepeat (partially); occurs as 5 respectively 6 successive sequences in ovine and bovine PrP*

MBP expressed without additional fusion protein (PrP), which frequently served as negative control in this study, was also of homogeneous quality (Fig 1, lane 7) and of expected size (MBP-β-gal α fragment, 50.8 kDa) which is somewhat larger (as expected) than MBP cleaved from the fusion protein after factor Xa digestion (42.5 kDa). Though we did not study its physical state, the soluble MBP-PrP product used is most likely in a monomeric or low oligomeric state representative for PrP^{C}, with a secondary structure that is high in alpha helix and random coil and low in beta-sheet [31, 32].

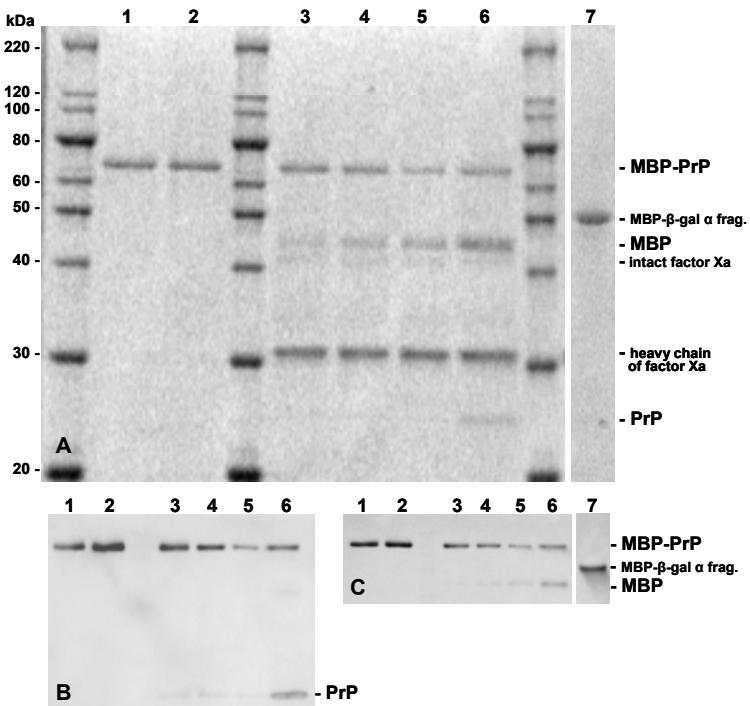


Figure 1. ***Analysis of MBP-PrP and MBP expression and MBP-PrP digestion by Factor Xa.*** *Lane 1 contains untreated MBP-PrP, whereas lane 2 contains a mock digestion of MBP-PrP. MBP-PrP was digested with 1% w/w factor Xa and during digestion samples were taken at 2, 4, 7 and 24 hours (lanes 3,4,5 and 6 respectively). All samples were run on SDS-PAGE and the gel was stained with Sypro Orange (total protein stain, panel A) before western blotting and subsequent immunodetection using either a PrP-specific monoclonal antibody (9A2, panel B) or MBP-specific monoclonal antibody (α-MBP, panel C). Expression of MBP, expressed from the pMAL-c2X vector with no insert (MBP-β-gal α fragment), was analyzed by Western Blot using either 9A2 or α-MBP (lane 7, panel A & C respectively).*

Binding domains of ovine PrP.

Using solid-phase arrays of 15-mer overlapping peptides systematically covering the whole mature part of PrP^C, MBP-PrP was allowed to bind with the peptide-array, with the prospect that this would yield information on interaction sites between its PrP moiety and the linear peptides. Indeed interaction between the individual PrP sequences (peptides) and MBP-PrP was sufficient for immunodetection, resulting in a reproducible binding pattern (Fig.2, line graph). This binding pattern, expressed in relative density values (Fig.2, column graph), was characterized by two distinct high binding areas (peptides 35-102 and 134-177 respectively) as well as some lower binding areas. Analysis of the correlating peptide sequences revealed that these areas usually were characterized by consensus sequences which suggested the existence of the following interaction domains for the mature part of PrP^C (Fig 3). Two consecutive binding peaks with peptides 22-28 + 29-33 (Fig.3A) have [33-**GWNTG**-37] (ovine protein sequence position used throughout) as their consensus domain, followed by two consecutive minor binding peaks with peptides 35-38 + 39-42 (Fig.3B) with [42-

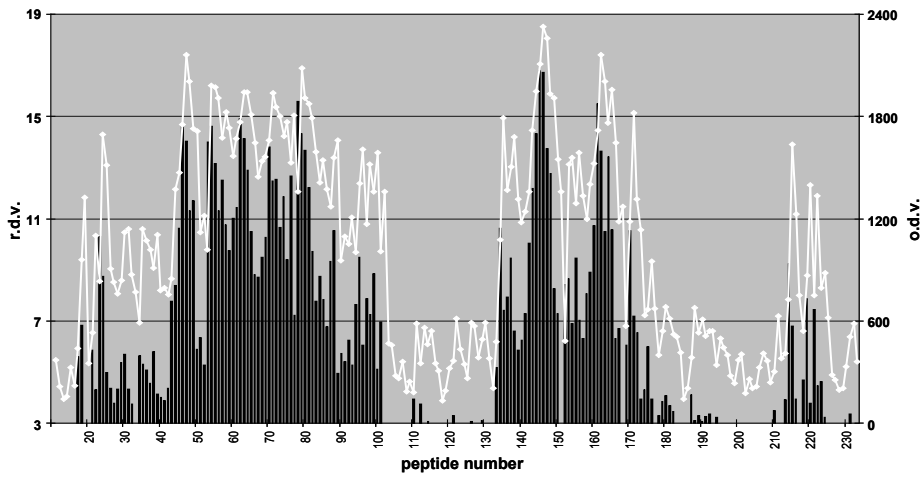


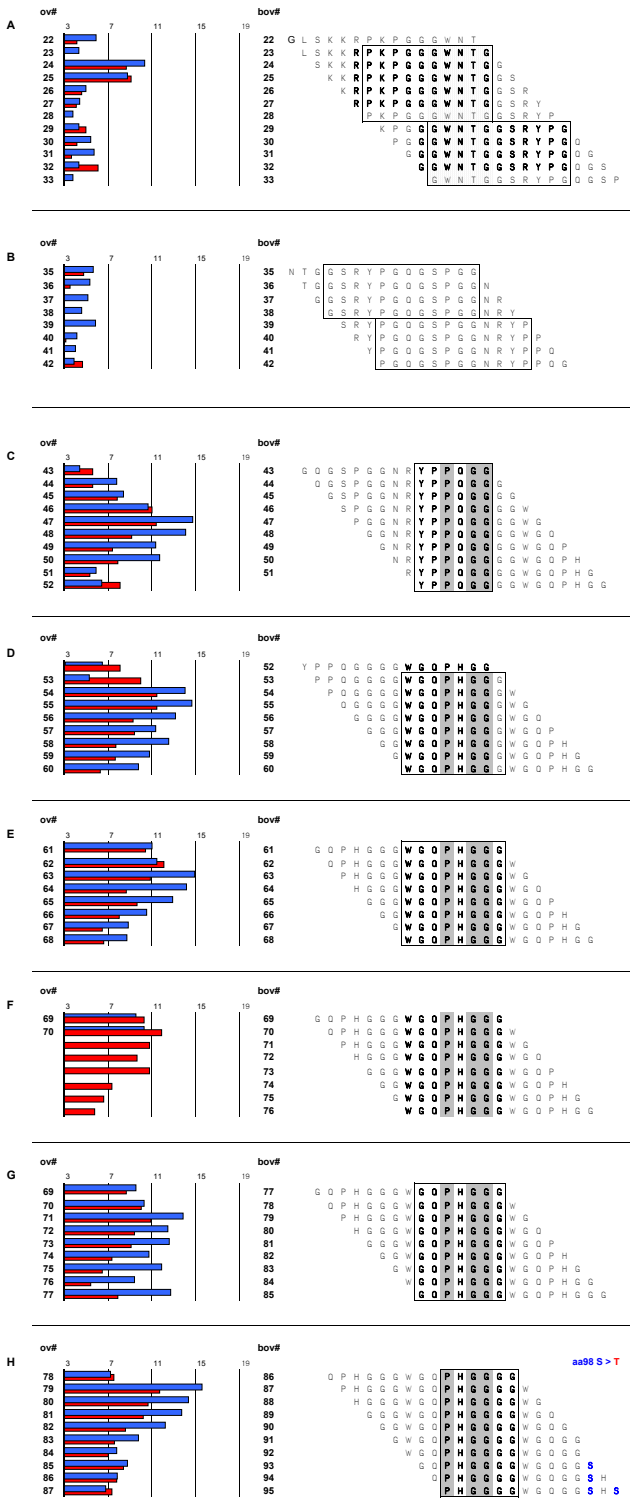
Figure 2. ***Peptide-array binding pattern of MBP-PrP.*** *Dual plot of MBP-PrP binding to the ovine PrP peptide-array. The relative density value (r.d.v.) was calculated by dividing the optical density value (o.d.v.) by the background and binding was considered relevant when at least 3 consecutive peptides showed binding values of at least 3 times the background. The unprocessed optical density values (left X-axis) of each peptide (Y-axis, peptide number) are plotted in the graph, while relative density values (right X-axis) of each peptide are plotted in the column graph.*

PGQGSPGG-49] as the common domain. The first high binding area is comprised of peptides 43-102, and encloses only two likely consensus domains: on the one hand peaks 43-52 (Fig.3C), 53-60 (Fig.3D), 61-68 (Fig.3E), 69-77 (Fig.3G) and 78-87 (Fig.3H) each recognized an octarepeat with [**PxGG**, x=Q or H] as the consensus domain and on the other hand peaks 90-93, 94-97, 98-102 (Fig.3I) with [102-**WNK**-104] as a common domain. The second high binding area is comprised of peptides 134-177 and encloses three likely consensus sequences. The shared sequence for peptides 134-136, 137-140 (Fig.3J) is [140-**PLIHFGNDY**-148], for peptides 141-151 (Fig.3K, AA152-154) and 153-155, 156-158, 59-164 (Fig.3L, AA165-167) is [**YYR**] and for peptides 165-168, 170-173, 174-177 (Fig.3M) is [177-**NFV**-179] respectively. The remaining lower binding areas also enclose three likely binding domain consensuses; [183-**VNITVKQHTVT**-193] for peptides 179-183 (Fig.3N), [192-**TTTTKGENFT**-202] for peptides 188-193 (Fig.3O) and [225-**SQAY**-228] for peptides 214-217, 219-221, 222-225 (Fig.3P).

Ovine versus bovine peptide-array.

To further assess the extent of the specificity of the binding pattern found, MBP-PrP was also tested against a bovine PrP peptide-array. This yielded a rather similar binding pattern compared to the results with ovine PrP peptide array but with some differences. The binding pattern on the bovine peptide-array (Fig.3, red bars) was compared to that on the ovine peptide-array (Fig.3, blue bars). As expected an extra octarepeat was found (Fig.3F), and of the six amino acid differences between the ovine -and bovine peptide arrays only two resulted in a difference in binding. Binding with the peptides containing the ovine to bovine substitutions S98A (ovine numbering used throughout, Fig.3H-I), S146A (Fig.3J) and Y158H (Fig.3K) were comparable on both the ovine and bovine peptide-array, whereas the S100G (Fig.3I) and Q189E (Fig.3N) did result in altered binding patterns. No binding was found with peptides containing the I208M substitution (data not shown). Some differences in binding without a direct apparent reason were observed. Differences in the relative level of binding was observed with peptides 165-177 (Fig.3M, bo# 173-785), 187-193 (Fig.3O, bo# 195-201) and 220-222 (Fig.3P, bo# 227-229), but these differences did not translate in differences in the determined consensus domains. However, binding with the array of bovine peptides 35-42 (Fig3.B) remained below the cutoff value (3 times background), whereas low binding with peptides 103-105 (Fig.3I) was observed with the bovine peptide-array but not with the ovine peptide-array.

Figure 3.



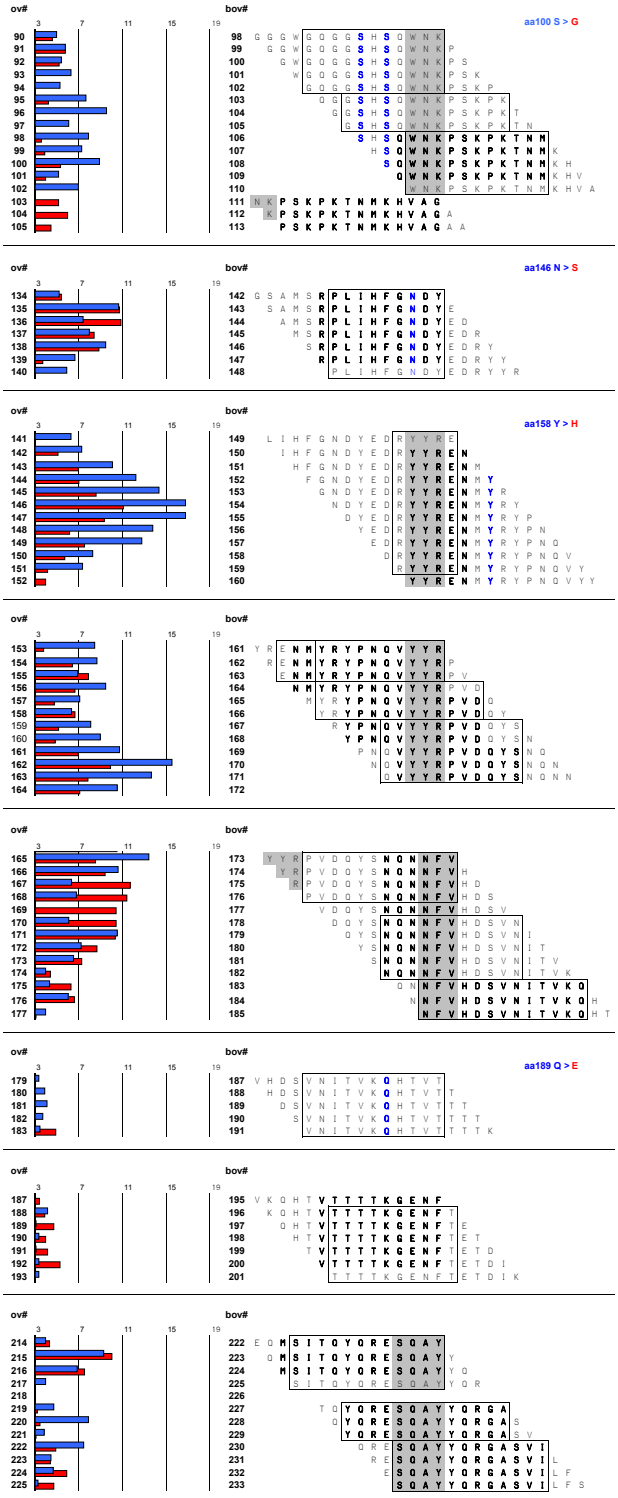

ov#
bov#
aa100 S > G
aa146 N > S
aa158 Y > H
aa189 Q > E

Figure 3 (previous pages). ***Correlation of relative binding pattern, peptide amino acid sequence and species variation between ovine and bovine PrP.*** *Horizontal bars represent r.d.v of each peptide in a binding region with the position of each peptide indicated for ovine (left numbers) and bovine (right numbers) peptide array. Amino acid sequences are the ovine peptide sequences of all peptides in designated binding regions. The consensus domains found within each binding region for the ovine sequence are boxed and the consensus domains of the corresponding peaks found with the bovine peptide-array are in bold font. Blue bars represent r.d.v of ovine peptide-array, red bars r.d.v. of bovine peptide-array. Substitutions in bovine PrP are mentioned at right top side of each panel (panels H, I, J, K,*

Peptide-array controls.

Several control tests were carried out to determine the viability of the peptide-array to obtain PrP-specific binding patterns. Only minor non-significant differences in binding pattern were seen as a result of varying concentration of MBP-PrP (except for the expected difference in optical density value [o.d.v.]), storage buffers for MBP-PrP, peptide synthesis methods, or peptide-array batches (Fig .4). Also, no significant binding was observed with each separate antibody or in the combination used for detection (thus in the absence of MBP-PrP). Furthermore no binding of MBP with the PrP peptide-array was observed. Interestingly the o.d.v. decreased after prolonged storage of MBP-PrP in PBS + 0.1% SB3-14. Further examination of the isolate showed that MBP-PrP had precipitated, indicating that interaction between the peptides and MBP-PrP only occurs when the latter is soluble. Furthermore, MBP-PrP was tested on an unrelated peptide-array containing overlapping peptides covering the sequence of VP2 of canine parvovirus yielding not any significant binding domains. All these controls confirm that binding of MBP-PrP to the PrP-peptides was as a result of the PrP moiety of MBP-PrP, and that this binding was PrP-specific.

Antibody blocking of peptide-array binding pattern.

To find a correlation with structural properties, the relative binding pattern of MBP-PrP on the peptide array was compared to the Kyte-Doolittle hydrophilicity plot of mature PrP^C revealing a high correlation between hydrophilic (exposed) regions of PrP^C and binding pattern regions (Fig.5). Even though the correlation was not absolute, it was necessary to determine if the binding pattern could be blocked with PrP-specific antibodies. Therefore several monoclonal antibodies with epitopes at different sites throughout PrP^C (Table 1, Fig.5) were tested. Pre-incubation (1 hour at room temperature) of MBP-PrP with all the tested PrP-specific monoclonal antibodies resulted in a concentration-

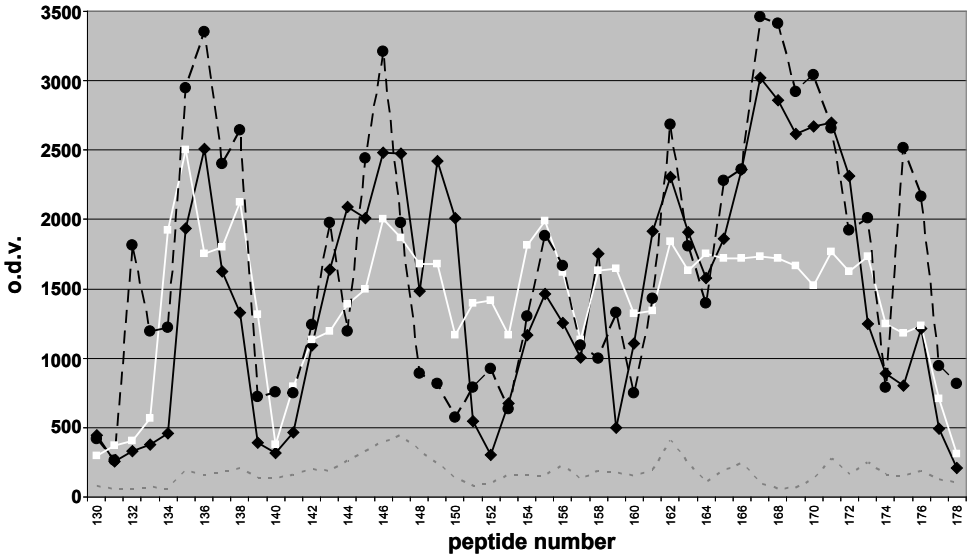


Figure 4. ***Peptide array controls.*** *Optical density value plot of MBP-PrP isolate 1 measured on minicard 1 (black line), MBP-PrP isolate 2 on minicard 1 (black dotted line), and MBP-PrP isolate 1 on minicard 2 (white line) and of MBP (background, grey dotted line) on peptide-array. For differences between isolates, compare black and white line. For differences between peptide synthesis batches compare black and black dotted line.*

dependant blocking of MBP-PrP binding over the whole set of PrP-peptides, albeit at different antibody concentrations (Table 1). No blocking of the MBP-PrP binding pattern occurred after pre-incubation with the unrelated Mycobacterium specific antibody M7. To ensure that blocking of the binding pattern is a result of immune-complex formation between antibody and MBP-PrP and not of incidental aspecific aggregation of MBP-PrP, a Mab-aggregation test for each PrP-specific antibody was carried out. Comparative amounts of MBP-PrP and antibody (necessary for blocking) were incubated. The soluble and insoluble fraction were separated by centrifugation at 20.000xg and analyzed on SDS-PAGE, resulting in 75 ± 16% of MBP-PrP and 85 ± 7% of antibody detected in the supernatant fraction (data not shown), indicating that if an immune-complex is formed this complex remains soluble. Therefore formation of a soluble immune-complex must be responsible for loss of binding in the peptide-array instead of diminished binding as a result of aspecific aggregation. In addition preliminary results indicate that some selected peptides are also capable of blocking MBP-PrP binding to the peptide-array, confirming that binding of MBP-PrP to the PrP-peptides was as a result of the PrP moiety of MBP-PrP and PrP-specific.

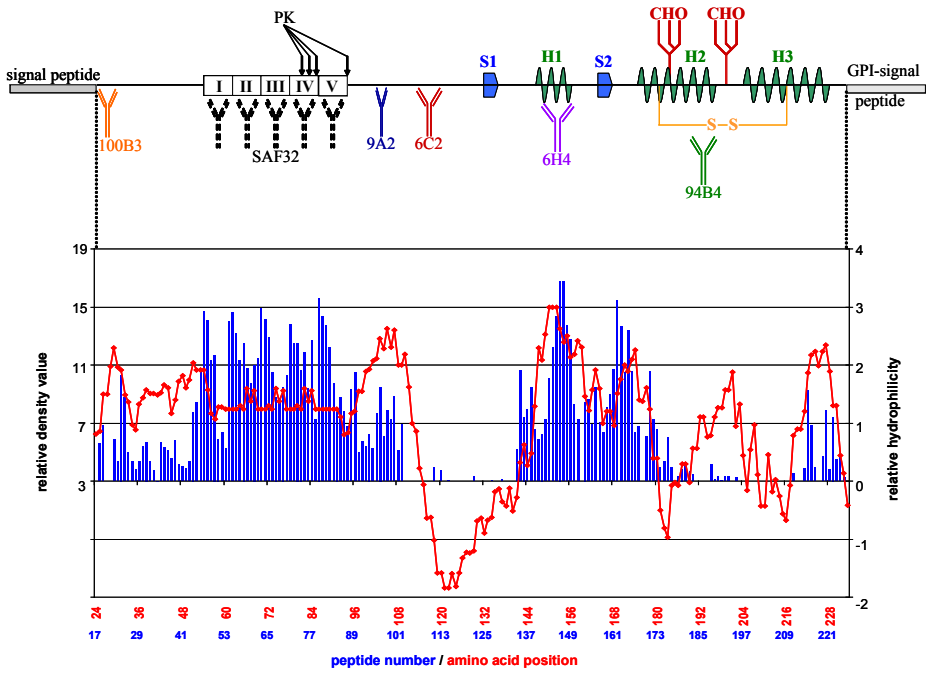


Figure 5. ***Overview of PrP^C secondary structures and antibody epitopes versus peptide-array binding pattern and Kyte-Doolittle hydrophilicity plot.*** *Schematic representation of PrP^C showing signal sequences, β-sheets (S1, S2), α-helices (H1, H2, H3), disulfide bridge site (S-S), glycosylation sites (CHO) and relative positions of the antibodies used in this study. The sequence of PrP is lined up with both the Kyte-Doolittle hydrophilicity plot (negative = hydrophobic and positive = hydrophilic) and the relative binding pattern found with the ovine peptide-array.*

MBP-PrP mediated detection (reverse detection) of PrP in Western blot.

To further confirm the specificity of the observed PrP-PrP interaction, MBP-PrP was used as a detector in Western blot to further study its affinity towards intact PrP^C in a brain homogenate. MBP-PrP could be used to detect recombinant His-tagged PrP (Fig.6B, left panel lane marked HP) and intact PrP in both scrapie positive and negative brain homogenates (Fig.6A, left panel lanes marked nt), albeit with a lower sensitivity under the standard Western blot conditions using monoclonal antibody 9A2 (Fig.6A, compare left and right panel). MBP-PrP seems to preferably detect the un-glycosylated PrP in the scrapie negative brain homogenate (in contrast to the scrapie positive homogenate). Correspondingly PNGase F treatment of the brain homogenates did not alter the capability of MBP-PrP to detect PrP in brain homogenates (Fig.6A, right panel lanes marked PF) even though detection with 9A2 showed decreased levels of

glycosylated PrP (Fig.6A, left panel lanes marked PF). MBP-PrP detection of PrP in the PNGase F treated scrapie positive homogenate still shows some detection of the different glycosylation forms (Fig.6A, right lower panel lane marked PF). Comparison of MBP-PrP detection of PrP in the PNGase F treated -and non-treated scrapie positive brain homogenate samples shows that after PNGase F treatment the amount of di-glycosylated PrP has decreased while mono-glycosylated PrP has increased. Therefore the detection of PrP glycoforms after PNGase treatment is not due to aspecific binding of MBP-PrP, but rather a result of incomplete de-glycosylation of PrP in this particular sample. In contrast, PK treatment of the brain homogenate abrogated MBP-PrP detection (Fig.6A, lanes marked PK) whereas immunodetection using monoclonal antibody 9A2 clearly shows the presence of PK-resistant PrP^{Sc} (Fig.6A, right panel lane marked PK) in the scrapie positive brain homogenate. As a control the same samples were tested in Western blot using free MBP, resulting in no detectable signal with either His-PrP or any of the brain homogenate samples (Fig.6, center panels). Only MBP-PrP (or MBP) was detected (Fig.6B, center panel, positive detection control), thus proving that the detection with MBP-PrP was PrP-specific.

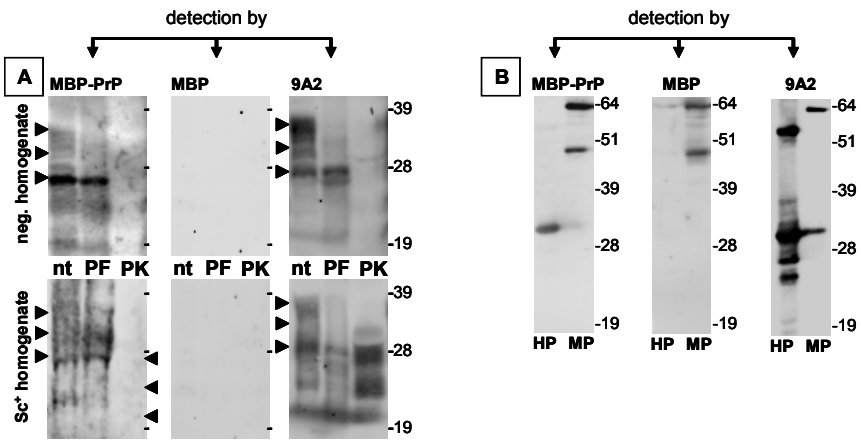


Figure 6. ***Interaction of recombinant MBP-PrP with various species of PrP in Western blot.*** *Samples containing either brain derived PrP [A] or recombinant derived PrP [B] were analyzed by SDS-PAGE and subsequent Western blotting. PrP was detected using MBP-PrP (left panels), MBP alone (center panels) or PrP-specific antibody 9A2 (right panels). Refer to the Methods section on "reverse detection assay" for specific details concerning immunodetection of membrane bound PrP. Aliquots from PrP^{Sc} negative ([A] upper panels) and PrP^{Sc} positive brain ([A] bottom panels) homogenates were either treated with PNGase F (PF) or proteinase K (PK) and compared to non-treated (nt) aliquots of brain homogenate. His-PrP ([B], lanes marked HP) was used as a positive PrP-control and MBP-PrP ([B] lanes marked MP) was used as a positive detection control. Arrow heads indicate the approximate position of the un-glycosylated, mono- and di-glycosylated PrP isoforms.*

Discussion.

Binding domains of ovine PrP^C.

Probing for possible PrP interaction domains using MBP-PrP and a solid-phase PrP peptide-array resulted in PrP specific interaction between specific PrP-sequences (peptides) and MBP-PrP. This probing revealed several likely interaction domains encompassed in two distinct high binding areas and some lower binding areas which will be discussed below in relation to structural features of PrP^C. Suggested properties in conversion, the species barrier and self-interaction sites as hypothesized in structural models will be discussed.

The first distinct high binding area contains two different interaction domain consensuses. The domain [**PHGG**] is repeated five times in the ovine peptide-array and six times in the bovine peptide-array (Fig.3C-H) and is part of the octarepeat sequence PHGGGWGQ, except for the first octarepeat (Fig.3C) where H is replaced with a Q. This substitution is considered neutral [33], confirmed by the lack of effect on the binding pattern found, even though Q is a polar residue and the weak positive charge of H is neutralized. The octarepeats are an epitope for antibodies inhibiting PrP^{Sc} propagation in cell culture [26]. This study further reveals domain [102-**WNK**-104] (Fig.3I) as a domain involved in PrP-PrP interaction. These three AAs are part of the epitope of the motif-grafted antibody containing mouse AA 89-112, which is capable of selective immuno-precipitation of infectivity [34].

The second high binding area contains 3 different interaction domain consensuses. This area also includes most of the polymorphisms found in sheep PrP^C. The domain [140-**PLIHFGNDY**-148] (Fig.3J) is situated between the first β-sheet (Fig.5, S1) and first α-helix (Fig.4, H1; the D and the Y are actually the first AA in α-helix 1) in PrP^C and is part of the epitope of the motif-grafted antibody containing mouse AA 136-158 (together with [152-**YYR**-154]), capable of selective immuno-precipitation of prion infectivity [34]. This domain encompasses the two amino acid positions which appear to fully control species-specific kinetics of PrP23-144 [35] by affecting amyloid fibril conformation, thus limiting which PrP^C molecule can adapt to the conversion seed. Therefore this domain is most likely involved in the species barrier and/or indirectly determines the susceptibility of sheep PrP to scrapie, maybe by influencing the accessibility of this domain and thus the adaptability of PrP^C to the conversion seed. Involvement of this region in adaptability between species was also concluded for human, mouse and hamster prions [35]. The [**YYR**] sequence occurs twice in the PrP sequence. AA [152-**YYR**-154] (Fig.3K) is

situated within the first α-helix (Fig.5, H1) and AA [165-**YYR**-167] (Fig.3L) is situated within the second β-sheet (Fig.5, S2) of PrP^{C}. The charged residues in the first α-helix, especially at residues 151, 152 and 154 (D,Y and R respectively) have also been shown to be a determinant of conversion [36]; substitutions of neutral amino acids or oppositely charged residues impaired conversion. Furthermore, [152-**YYR**-154] (together with [140-**PLIHFGNDY**-148]) is part of the of the epitope of the motif-grafted antibody containing mouse AA 136-158, capable of selective immuno-precipitation of prion infectivity [34]. The [**YYR**] domain has also been described as the epitope of an antibody that selectively recognizes PrP^{Sc} [37], and is part of several antibody (fragment) epitopes that prevent scrapie infection in tissue culture [18, 19, 23, 25, 26] or in a mouse model [24]. The putative domain [177-**NFV**-179] is the common AA sequence of three consecutive peaks of which the fist two peaks encompass amino acids of a peptide (corresponding to AA 163-176) that inhibits in vitro conversion [15]. However, because of the third peak it is more likely that this domain is involved in self-interaction.

The remaining lower binding areas are more difficult to interpret. The domain consensus for the peptides 22-33 (Fig.3A) seems to be [33-**GWNTG**-37], but may be a result of a cross-interaction with WN, instead of binding to domain [102-**WNK**-104] within the first high binding area. Binding with domain consensus [42-**PGQGSPGG**-49] (Fig.3B) is relatively low and may likely be due to cross-interaction with the proline (P) and two consecutive glycines (G) of these peptides, instead of binding to the consensus octarepeat domain [**PxGG**]. Binding with the domains [183-**VNITVKQHTVT**-193] (Fig3.N) and [192-**TTTTKGENFT**-202] (Fig.3O) is also (very) low and these domains comprise the second helix in PrP^{C} (Fig.5, H2) which in turn may explain the relative low binding due to the structured nature of this part of the protein. It is not clear what the importance is of these domains, but part of the latter domain is contained within a peptide (corresponding to AA 197-220) that inhibits in vitro conversion [15] as well. The last low binding domain found is [225-**SQAY**-228], which (together with [**YYR**]) is part of the non-linear epitope of the PrP^{Sc} specific monoclonal antibody 15B3 [18].

Several studies identified antibodies [18, 19, 23-26] or peptides [15] able to inhibit prion propagation. The binding domains found with the peptide-array containing AA corresponding with these antibody epitopes or peptides may also be (in)directly involved in conversion. However, the inhibitory effects of antibodies seems simply due to steric hindrance preventing any PrP interaction at all (also confirmed by our peptide-array blocking results), making the binding domains corresponding to the conversion inhibiting peptides (Fig.3M, peak1+2 and Fig.3O) the more likely candidate domains influencing conversion.

Our data are in line with a theoretical two rung β-helical model described by Langedijk *et al.* [38], which tries to explain how AA sequence and secondary structure could explain strain properties and the species barrier. The binding domains found by peptide-array are all exposed in the periphery of the proposed fibril. The first distinct high binding domain makes up the incoming protein chain, whereas the second distinct high binding area forms the loop connecting the two-rung β-helical core with the α-helices (the outgoing protein chain). Furthermore, no binding is observed between the two distinct high binding areas and the AA sequence of these peptides correlate with the predicted two rung β-helical core.

Our data provide insight in the possible interaction domains of PrP^{C} with itself or PrP^{Sc}, and most of the domains identified are likely to be involved in PrP^{C} self-interaction. This may involve dimerisation of PrP^{C} and/or formation of a trimer of three PrP^{C} molecules as suggested in the theoretical two-rung β-helical model for PrP stacking during PrP^{Sc} induced fibrillization [38]. If one of the identified high binding domains is of influence on conversion, this possibly is exerted during pre-oligomerisation, which is an inefficient process. On the other hand, a direct effect on PrP^{C}-PrP^{Sc} binding can not be excluded.

Ovine versus bovine peptide-array.

In addition to the ovine peptide-array, a similar array of overlapping 15-mer peptides bovine peptides was used. Since there are several sequential differences between bovine and ovine PrP this may be of influence on the overall binding pattern found with MBP-PrP (ovine PrP). Generally it seems that binding of MBP-PrP is somewhat less efficient (strong) with the bovine peptide-array compared to binding with the ovine peptide-array. However, this had no significant effects on the relative binding pattern. In the amino acid sequence of bovine PrP there is an extra octarepeat as well as six amino-acid substitutions. Only the largest differences will be discussed here. The non-discussed differences may well be the result of minor methodological variations when producing the peptide-arrays.

As expected, an extra octarepeat (Fig.3F) was evident that confirmed the octarepeats consensus binding domain [PHGG]. Only two out of six amino acid substitutions were of influence on the binding pattern. At first glance the amino acid substitutions at AA 98 and 100 (sheep numbering used throughout) are seemingly both of influence on binding with peptides 93-97 (Fig.3I, bo# 98-113), allowing detection of the consensus binding domain [102-**WNK**-104] only when these AA were no longer present in the peptides. The supposedly neutral substitution of glycine for serine at AA 100 is most

likely responsible for the observed differences in binding and may be attributed to the greater conformational flexibility of G, affecting availability of other AA's for interaction with MBP-PrP. Taking this in account, binding with peptides 90-92 should be a result of cross-binding with two consecutive Glycines present in these peptides in stead of binding with the consensus octarepeat domain. Glutamatic acid only contains an oxygen in place of the amido group in glutamine and therefore these AA's are considered readily interchangeable [33]. However we observed that the substitution of glutamatic acid for glutamine at AA 189 did affect binding (Fig.3N) on the bovine peptide-array with peptides 179-183; Both AA's are polar, but where glutamine interacts with other polar or charged atoms with its polar side chains, glutamatic acid is negatively charged and is frequently involved in salt-bridges and/or glutamatic acid interacts with positive charged AA's to form hydrogen bonds. These differences in AA reactivity are most probably responsible for the observed difference in binding between ovine and bovine PrP peptide sequences.

Analysis and comparison of the relative (consensus) ovine -and bovine peptide-array revealed that the detection of potential PrP-PrP interaction domains using this method is robust as well as sensitive to differences in structural flexibility and/or amino acid differences. Therefore, this peptide-array approach provides a possibly valuable tool to assess the influence of disease associated polymorphisms on available interaction domains and to test for PrP-PrP binding inhibitors potentially useful in therapy (i.e. antibodies or peptides).

Antibody blocking of peptide-array binding pattern.

All monoclonal antibodies (mab) recognizing PrP (Table 1, Fig.5) are capable of blocking the complete binding pattern. Differences in the antibody concentration necessary for complete blocking are likely due to epitope availability and/or affinity for PrP. Complete blocking of the binding pattern can best be explained by steric hindrance of the antibodies preventing any interaction. It has been described that binding of a monoclonal antibody at the N-terminus of human PrP influences epitope availability at the C-terminus [39] and similar events may also attribute to completely abolishing the binding pattern. Furthermore, structural studies of PrP^{Sc} that resulted in prion propagation/fibrillization models [38, 40, 41], suggest that PrP-PrP interaction depends on the structure of the whole protein (not just the trimer or dimer core). These findings corroborate the notion that antibodies inhibiting prion propagation probably do so by preventing the interaction between PrP^{C} and PrP^{Sc} or between PrP^{C} molecules themselves in the pre-oligomerisation phase.

MBP-PrP mediated detection (reverse detection) of PrP in Western blot.

By using MBP-PrP as the detecting agent in Western blot we showed that binding of MBP-PrP to the peptide-arrays is PrP specific and indicative of a PrP-PrP interaction. MBP-PrP seems to preferably detect un-glycosylated PrP^{C} in the scrapie negative brain homogenate. The exact reason for this preference is unclear, but does not seem to be due to the lack of glycosylated isoforms in the brain homogenate (except after de-glycosylation with PNGase F) as shown by immuno-detection with 9A2. It may be possible that the determined interaction domains do not (only) interact with the same amino acid motif (self-self), but that an intramolecular cross-interaction between different domains can also occur. The peptide-array data has revealed two high binding areas, one of which contains the N-terminal octarepeats and the other contains the first α-helix, second β-sheet up to the second α-helix directly adjacent to the glycosylation sites (Fig.5). When PrP^{C} in the homogenate is glycosylated this may sterically hinder binding of MBP-PrP to PrP^{C}. Glycosylation is suggested to have a role in prion strain maintenance and the species barrier [42] by modulating the fidelity of interaction, which may explain the favorable binding of un-glycosylated PrP by MBP-PrP (which is also un-glycosylated, indicating that binding preferably occurs between compatible glycosylated molecules) in the scrapie negative homogenate. However, in the scrapie positive brain homogenate detection of PrP by MBP-PrP is more diffuse and might suggest that MBP-PrP detection of PrP in Western blot is due to interaction with both full-length PrP^{C} as well as full length denatured PrP^{Sc}, which is comparable to PrP^{C}. Alternatively, PrP^{Sc} might be partly endogenously truncated resulting in more heterogeneous binding of MBP-PrP to all glycosylation forms. In contrast, MBP-PrP detects un-glycosylated PrP as well as both mono -and di-glycosylated PrP in the scrapie positive homogenate, even though un-glycosylated PrP usually is the lesser component in the PrP-triplet of scrapie sheep brain samples, which may be indicative for preferable binding of un-glycosylated PrP in the scrapie positive homogenate. However interpretation of these results is difficult and in order to elucidate the precise effects of glycosylation on binding between PrP molecules, interaction should be studied under more native conditions. When proteinase K treatment was applied MBP-PrP did not detect PK-resistant PrP^{Sc}. This indicates that in order for MBP-PrP to detect PrP in brain homogenates (under the conditions used) full length (at least containing the high binding area with octarepeats) PrP molecules are required. It may be hypothesized that the first high binding area containing the octarepeats aids in stabilization of PrP self-interaction, perhaps by intramolecular interaction with other mapped interaction domains. This extra stabilization in turn

allows further immunodetection in Western blot (under the conditions used). These results combined with the results obtained by peptide-array analysis support the concept of self-interactive domains of PrP^{C}.

Conclusions.

In summary, probing for possible interaction domains in PrP using a solid phase PrP peptide-array revealed that specific interactions take place between individual PrP molecules. Ten possible consensus binding domains were found, which includes one domain that likely is due to a cross-reaction with the octarepeat domain consensus –and for two domains it remains unclear what their importance is. The remaining seven domains are most likely involved in PrP^{C} self-interaction. Furthermore, MBP-PrP was also capable to specifically bind to full length PrP^{C} and PrP^{Sc} bound PrP^{C} in Western blot confirming PrP-PrP specific interaction. Together these results indicate that in addition to direct PrP^{C}-PrP^{Sc} interactions several other molecular interactions between PrP^{C} molecules/sequences themselves may also be possible, facilitating initial steps in the oligomerisation process.

The PrP peptide-array may additionally facilitate in gaining insight into effects of disease associated polymorphisms in PrP on PrP-PrP binding, and the subsequent molecular conversion of PrP^{C} into PrP^{Sc}. The (self-) interaction domains described here may ultimately prove useful in the design of therapeutics interfering in the PrP-PrP binding process.

Methods.

MBP-PrP construct.

In order to obtain the PrP gene suitable for cloning into the pMAL Protein Fusion and Purification System (New England Biolabs), the mature part of the sheep PrP (ARQ) open reading frame (ORF) was PCR amplified using primers ShBo-F-DraI (GGTGGTTTTAAAA-AGCGACCAAAACCTGG) and Sh-R-STOP (GGTGGTCTATGCCCCCCTTTGGTAATA-AGCC). The resulting PrP (AA25-233), without its N -and C-terminal signal sequences, was cloned into a general TA-cloning vector (Invitrogen) and sequenced to exclude PCR artifacts. The PrP fragment was subsequently sub cloned using *Dra*I and *Eco*RI into the pMAL-c2X expression vector, resulting in the maltose binding protein (MBP) fusion to the N-terminus of PrP (MBP-PrP).

MBP-PrP expression and purification.

Expression and purification by affinity chromatography was performed as described in the manual of the pMAL Protein Fusion and Purifications System (method I; New England Biolabs) To improve binding of MBP-PrP to prevent formation of interchain disulfide upon lysis (as suggested in the protocol), β-mercaptoethanol was added. Quantity and quality of the eluted MBP-PrP was determined by SDS-PAGE (12% NuPAGE, Invitrogen). After separation the gel was either stained with Sypro Orange (total protein stain, Molecular probes) or analyzed by Western blotting and immunodetection of MBP-PrP with polyclonal antiserum R521-7 specific for PrP. To obtain MBP for cross-reaction aspecificity tests, the pMAL-c2x expression vector without insert was expressed and purified as described above.

Peptide-array analysis.

Synthesis of complete sets of overlapping 15-mer peptides were carried out on grafted plastic surfaces, covering the ovine or bovine PrP amino acid sequence of mature PrP (residues 25-234 of ovine and 25-242 of bovine PrP) [43]. The plastic surface consisted of 455, 3µl wells on a credit-card size plastic (minicard) carrier. Peptide-arrays covering the ovine or bovine PrP amino acid sequence of mature PrP were custom synthesized through two different synthesis techniques: either all peptides of the array were synthesized in situ to the grafted plastic surface by step-by-step amino acid coupling or the peptides were synthesized separately and coupled as complete 15-mer peptides to each well at their C-terminus [44-46]. In subsequent ELISA analyses on the minicards, MBP-PrP was incubated as an antigen followed by immuno-screening with mouse anti-MBP monoclonal antibody (Mab) obtained from New England Biolabs and rabbit anti-mouse-IgG-peroxidase, or a rabbit anti-MBP Mab and swine anti-rabbit-IgG-peroxidase (from DAKO, Denmark). Blocking studies were performed by pre-incubating the MBP-PrP with a

PrP-specific Mab before incubating the mixture as the antigen on the minicard. The background was determined by calculating the mean value of 20 peptides with low density values of which at least 5 peptides were in consecutive order. The relative density value (r.d.v.) was calculated by dividing the optical density value (o.d.v.) by the background and binding was considered relevant when at least 3 consecutive peptides showed binding values of at least 3 times the background.

Production of monoclonal antibody 6C2.

Monoclonal antibody 6C2 was newly prepared using PrP-knockout mice immunized with peptide KTNMKHVAGAAAAG (ovine PrP109-122), conjugated through a cysteine at its C-terminus to Keyhole limpet hemocyanine, using previously described procedures for synthesis and screening [47]. In ELISA and Western blot antibody 6C2 binds respectively to ovine –and bovine recombinant PrP and ovine –and bovine PrP^{res} at the approximate residues HVAGAAA as determined by peptide mapping analysis using an ovine peptide-array.

Antibody aggregation test.

Each reaction contained 500ng MBP-PrP and monoclonal antibody in PBS containing 0.05% Tween80. For each antibody the inhibitory concentration as well as an excess concentration (max. 25µg/ml) was tested. The reaction was incubated for 1 hour at room temperature and subsequently centrifuged for 30 minutes at 20,000xg. Most of the supernatant was transferred to a new tube except approximately 3-5µl to prevent disturbance of the pellet. The pellet fraction was dissolved in 0.1% SDS by sonification. Both fractions were subjected to methanol precipitation and analyzed by SDS-PAGE (12%, NuPAGE), Western blot and immunodetection using either R521-7 (rabbit anti-PrP serum [48]) and swine anti-rabbit-IgG-peroxidase (PrP detection) or rabbit anti-mouse-IgG-peroxidase (antibody detection). The relative amount of MBP-PrP or antibody band(s) detected as fluorescent signal (f.s.) in Western blot was determined by using the ECF substrate for detection and the Molecular Dynamics ImageQuant software for quantification. Subsequently the mean percentages of MBP-PrP or antibody in the soluble (supernatant) fraction ($f.s.^{sup}/[f.s.^{sup}+f.s.^{pel}]$) were calculated.

PrP^{Sc} purification and analysis.

PrP^{Sc} was isolated from brain tissue of clinically ill scrapie sheep. PrP genotypes were determined by Sanger sequencing of the full PrP-ORF as described before [72]. PrP^{Sc} was purified by ultracentrifugational pelleting from sarcosyl-homogenated brains as described previously [3, 50]. The final pellets were sonicated in phosphate-buffered saline containing 1.0% SB 3-14. Yields of PrP^{Sc} were quantified by SDS-PAGE (12% NuPAGE) and Western blotting using antiserum R521-7.

Reverse detection assay.

Confirmed scrapie positive and negative 10% sheep brain homogenates were digested with either proteinase K (PK) or PNGase F and compared to the non-treated samples. A separate aliquot of 10% brain homogenate was treated with 35 µg/ml PK for 1 hour at 37°C. Another aliquot was denatured by adding $^{1}/_{10}$ volume denaturing buffer (5% sodium dodecyl sulphate and 10% β-mercaptoethanol in 20 mM Tris-HCl– 150 mM NaCl– 2 mM EDTA [pH 7.5]) for and subsequent heating 10 min. at 96°C. This aliquot was de-glycosylated in the presence of 1000U PNGase F/ml for at least 36 hours at 37°. Untreated, PK treated –and PNGase F treated brain homogenates samples were analyzed by SDS-PAGE and Western blot. As positive controls His-PrP (positive PrP control) and MBP-PrP (positive detection control) were included. Reverse detection of PrP was accomplished by incubating the Western blot for 1 hour at room temperature with approximately 2ng/µl MBP-PrP followed by immunodetection using mouse anti-MBP monoclonal antibody and rabbit anti-mouse-IgG-peroxidase (RAMPO). PrP was also detected on Western blot using the PrP-specific monoclonal antibody 9A2 and RAMPO. To determine if detection is PrP specific, a Western blot was carried out with MBP alone instead of MBP-PrP.

Acknowledgements.

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References.

1. DeArmond, S.J. and S.B. Prusiner, *Perspectives on prion biology, prion disease pathogenesis, and pharmacologic approaches to treatment.* Clin Lab Med, 2003. 23(1): p. 1-41.
2. Bossers, A., et al., *In vitro conversion of normal prion protein into pathologic isoforms.* Clin Lab Med, 2003. 23(1): p. 227-47.
3. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.
4. Raymond, G.J., et al., *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans.* Nature, 1997. 388(6639): p. 285-8.
5. Bossers, A., *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie*. 1999, University of Utrecht.
6. Bossers, A., R. de Vries, and M.A. Smits, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol, 2000. 74(3): p. 1407-14.
7. Raymond, G.J., et al., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo J, 2000. 19(17): p. 4425-30.
8. Kocisko, D.A., et al., *Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier.* Proc Natl Acad Sci U S A, 1995. 92(9): p. 3923-7.
9. Bessen, R.A., G.J. Raymond, and B. Caughey, *In situ formation of protease-resistant prion protein in transmissible spongiform encephalopathy-infected brain slices.* J Biol Chem, 1997. 272(24): p. 15227-31.
10. Dubois, M.A., et al., *Multiplicative genetic effects in scrapie disease susceptibility.* C R Biol, 2002. 325(5): p. 565-70.
11. Tranulis, M.A., *Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep.* Apmis, 2002. 110(1): p. 33-43.
12. Sabuncu, E., et al., *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol, 2003. 77(4): p. 2696-700.
13. Rigter, A. and A. Bossers, *Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion.* J Gen Virol, 2005. 86(Pt 9): p. 2627-34.
14. Holscher, C., H. Delius, and A. Burkle, *Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation.* J Virol, 1998. 72(2): p. 1153-9.

15. Horiuchi, M., et al., *Inhibition of interactions and interconversions of prion protein isoforms by peptide fragments from the C-terminal folded domain.* J Biol Chem, 2001. 276(18): p. 15489-97.
16. Peretz, D., et al., *A conformational transition at the N terminus of the prion protein features in formation of the scrapie isoform.* J Mol Biol, 1997. 273(3): p. 614-22.
17. Chabry, J., B. Caughey, and B. Chesebro, *Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides.* J Biol Chem, 1998. 273(21): p. 13203-7.
18. Korth, C., et al., *Prion (PrPSc)-specific epitope defined by a monoclonal antibody.* Nature, 1997. 390(6655): p. 74-7.
19. Peretz, D., et al., *Antibodies inhibit prion propagation and clear cell cultures of prion infectivity.* Nature, 2001. 412(6848): p. 739-43.
20. Scott, M., et al., *Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes.* Cell, 1993. 73(5): p. 979-88.
21. Priola, S.A. and B. Chesebro, *A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells.* J Virol, 1995. 69(12): p. 7754-8.
22. Priola, S.A. and V.A. Lawson, *Glycosylation influences cross-species formation of protease-resistant prion protein.* Embo J, 2001. 20(23): p. 6692-9.
23. Enari, M., E. Flechsig, and C. Weissmann, *Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody.* Proc Natl Acad Sci U S A, 2001. 98(16): p. 9295-9.
24. Heppner, F.L., et al., *Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies.* Science, 2001. 294(5540): p. 178-82.
25. Beringue, V., et al., *PrPSc binding antibodies are potent inhibitors of prion replication in cell lines.* J Biol Chem, 2004. 279(38): p. 39671-6.
26. Perrier, V., et al., *Anti-PrP antibodies block PrPSc replication in prion-infected cell cultures by accelerating PrPC degradation.* J Neurochem, 2004. 89(2): p. 454-63.
27. Gasset, M., et al., *Perturbation of the secondary structure of the scrapie prion protein under conditions that alter infectivity.* Proc Natl Acad Sci U S A, 1993. 90(1): p. 1-5.
28. Solforosi, L., et al., *Toward Molecular Dissection of PrPC-PrPSc Interactions.* J Biol Chem, 2007. 282(10): p. 7465-71.
29. Biacabe, A.-G., et al., *H-Type Bovine Spongiform Encephalopathy.* Prion, 2007. 1(1): p. in press.
30. Thuring, C.M., et al., *Immunohistochemical distinction between preclinical bovine spongiform encephalopathy and scrapie infection in sheep.* J Comp Pathol, 2005. 132(1): p. 59-69.

31. Rezaei, H., et al., *Amyloidogenic unfolding intermediates differentiate sheep prion protein variants.* J Mol Biol, 2002. 322(4): p. 799-814.
32. Riek, R., et al., *NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231)*, in *FEBS Lett*. 1997. p. 282-8.
33. Betts, M.J. and R.B. Russel, *Amino acid properties and consequences of substitutions*, in *Bioinformatics for Geneticists*, M.R. Barns and I.C. Gray, Editors. 2003, John Wiley & sons.
34. Moroncini, G., et al., *Motif-grafted antibodies containing the replicative interface of cellular PrP are specific for PrPSc.* Proc Natl Acad Sci U S A, 2004. 101(28): p. 10404-9.
35. Surewicz, W.K., E.M. Jones, and A.C. Apetri, *The emerging principles of Mammalian prion propagation and transmissibility barriers: insight from studies in vitro.* Acc Chem Res, 2006. 39(9): p. 654-62.
36. Norstrom, E.M. and J.A. Mastrianni, *The charge structure of helix 1 in the prion protein regulates conversion to pathogenic PrPSc.* J Virol, 2006. 80(17): p. 8521-9.
37. Paramithiotis, E., et al., *A prion protein epitope selective for the pathologically misfolded conformation.* Nat Med, 2003. 9(7): p. 893-9.
38. Langedijk, J.P., et al., *Two-rung Model of a Left-handed beta-Helix for Prions Explains Species Barrier and Strain Variation in Transmissible Spongiform Encephalopathies.* J Mol Biol, 2006. 360(4): p. 907-920.
39. Li, R., et al., *Identification of an epitope in the C terminus of normal prion protein whose expression is modulated by binding events in the N terminus.* J Mol Biol, 2000. 301(3): p. 567-73.
40. Wille, H., et al., *Structural studies of the scrapie prion protein by electron crystallography.* Proc Natl Acad Sci U S A, 2002. 99(6): p. 3563-8.
41. Govaerts, C., et al., *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A, 2004. 101(22): p. 8342-7.
42. Lawson, V.A., et al., *Prion protein glycosylation.* J Neurochem, 2005. 93(4): p. 793-801.
43. Goldmann, W., et al., *Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon.* J Gen Virol, 1991. 72 (Pt 1): p. 201-4.
44. Timmerman, P., et al., *Mapping of a discontinuous and highly conformational binding site on follicle stimulating hormone subunit-beta (FSH-beta) using domain Scan and Matrix Scan technology.* Mol Divers, 2004. 8(2): p. 61-77.
45. Piccardo, P., et al., *An antibody raised against a conserved sequence of the prion protein recognizes pathological isoforms in human and animal prion diseases, including Creutzfeldt-Jakob disease and bovine spongiform encephalopathy.* Am J Pathol, 1998. 152(6): p. 1415-20.

46. Geysen, H.M., R.H. Meloen, and S.J. Barteling, *Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid.* Proc Natl Acad Sci U S A, 1984. 81(13): p. 3998-4002.

47. Langeveld, J.P., et al., *Rapid and discriminatory diagnosis of scrapie and BSE in retropharyngeal lymph nodes of sheep.* BMC Vet Res, 2006. 2: p. 19.

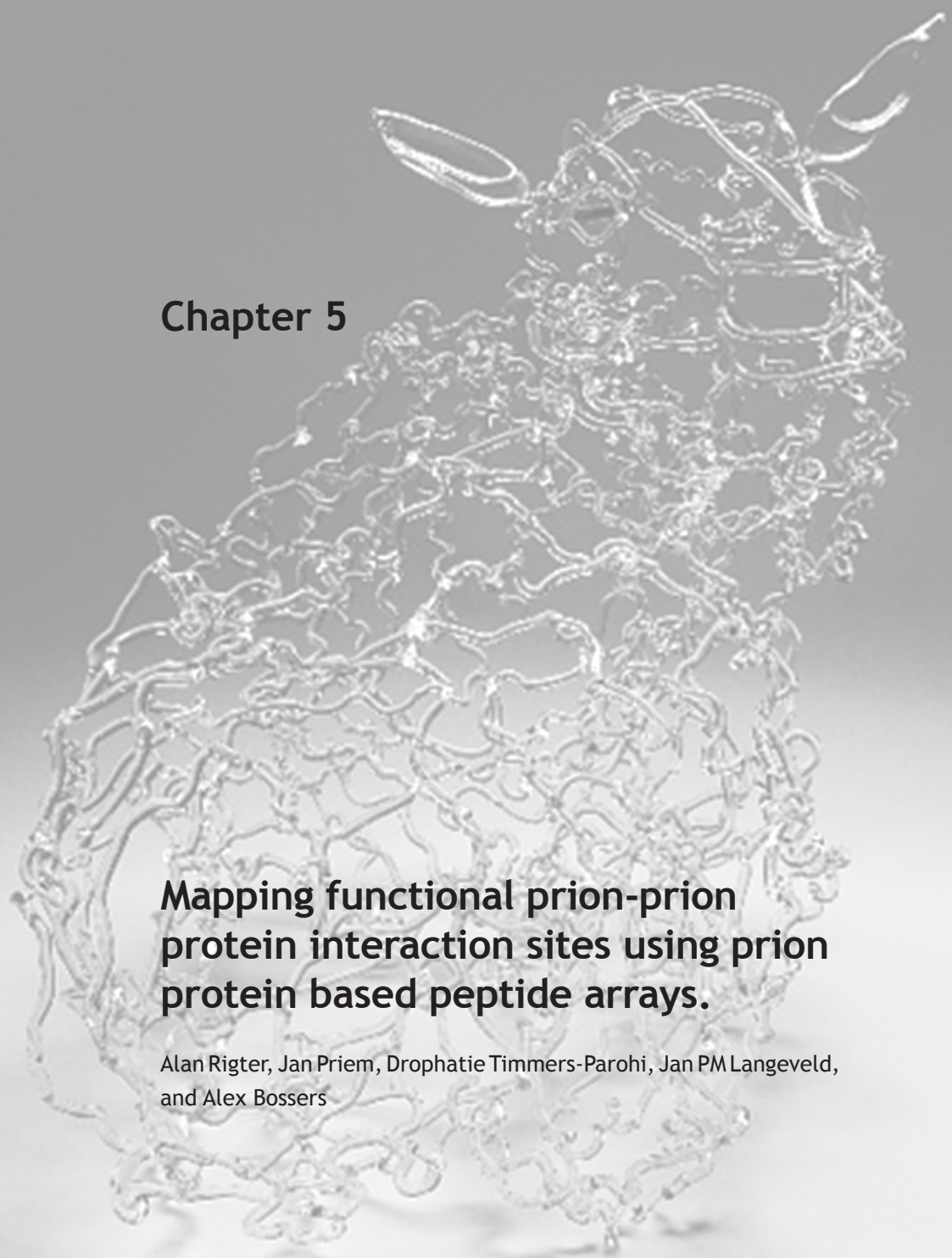
48. van Keulen, L.J., et al., *Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie.* Vet Pathol, 1995. 32(3): p. 299-308.

49. Bossers, A., et al., *PrP genotype contributes to determining survival times of sheep with natural scrapie.* J Gen Virol, 1996. 77 (Pt 10): p. 2669-73.

50. Caughey, B., et al., *Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state.* Chem Biol, 1995. 2(12): p. 807-17.

51. Feraudet C, et al., *Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells.* J Biol Chem 2005, 280(12): p.11247-11258.

52. Thuring CM, et al., *Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein.* J Clin Microbiol 2004, 42(3): p.972-980.



Chapter 5

Mapping functional prion-prion protein interaction sites using prion protein based peptide arrays.

Alan Rigter, Jan Priem, Drophatie Timmers-Parohi, Jan PM Langeveld, and Alex Bossers

Methods in Molecular Biology; Peptide Microarrays. 2009; 570:257-71. Review.

Mapping functional prion-prion protein interaction sites using prion protein based peptide arrays.

Alan Rigter, Jan Priem, Drophatie Timmers-Parohi, Jan PM Langeveld, and Alex Bossers

Abstract.

Protein-protein interactions are at the basis of most if not all biological processes in living cells. Therefore, adapting existing techniques or developing new techniques to study interactions between proteins are of importance in elucidating which amino acid sequences contribute to these interactions. Such new insights may in turn lead to improved understanding of the processes underlying disease and possibly provide the basis for new therapeutic approaches. Here we describe the novel use of an ovine prion protein based peptide-array normally used for determining prion-specific antibody epitopes, with the prospect that this would yield information on interaction sites between its PrP moiety and the ovine prion protein derived linear peptides. This adapted application of the peptide-array shows, by incubating the mature part of ovine (ARQ) PrP^{C} fused to maltose binding protein (MBP), binding with between the PrP moiety and the ovine prion derived peptides occurs and indicates that several specific self-interactions between individual PrP molecules can occur. Hereby illustrating that this adapted application of a peptide-array is a viable method to further specify which distinct amino acid sequences are involved in protein-protein interaction.

1. Introduction.

Central in many biological processes is de interaction between proteins. This encompasses interaction with other as well as 'self' proteins, resulting in a multitude of effects. One example is signal transduction, in which extracellular signals are conferred to the inside of a cell by protein-protein interactions of the signaling molecules. Another is the long term interaction between proteins as part of a protein complex, in which one protein helps another from one to another cellular compartment (i.e. importins). Also brief interactions between proteins occur, resulting in modification of the target protein (i.e. post-translational modification), which in itself can change protein-protein interactions. Thus, protein-protein interactions are of importance for most if not all biological processes in living cells. Therefore, study of these interactions and elucidating the contributing amino acid sequences involved in these interactions may lead to improved understanding of the processes underlying disease and may provide the basis for new therapeutic approaches.

The common event in transmissible spongiform Encephalopathies (TSEs) or prion diseases is the conversion of host-encoded protease sensitive cellular prion protein (PrP^{C}) into strain dependent isoforms of scrapie associated protease resistant isoform (PrP^{Sc}) of prion protein (PrP). Formation of PrP^{Sc} is a posttranslational process and involves refolding (conversion) of the host-encoded prion protein (PrP^{C}) into partially protease resistant forms (PrP^{Sc}) [1].

These processes are determined by similarities as well as strain dependent variations in the PrP structure [2-11] Selective self-interaction between PrP molecules is the most probable basis for initiation of these processes, potentially influenced by chaperone molecules; however the mechanisms behind these processes are far from understood. Here we describe the utilization of a peptide-array consisting of 15-mer overlapping peptides systematically covering the whole mature part of the cellular prion protein (Fig.1) in order to elucidate the interaction domains involved in self-interaction of PrP^{C} [12]. To this end, an ovine PrP peptide-array consisting of 15-mer overlapping peptides was probed with recombinant sheep PrP^{C} fused to maltose binding protein (MBP-PrP).

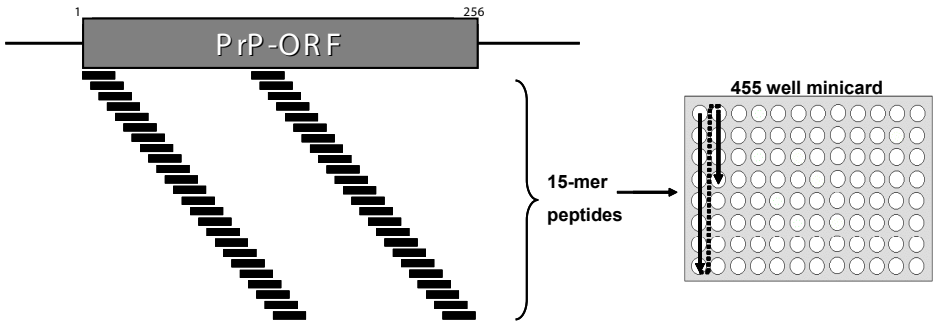


Figure 1. ***Basic setup of the prion peptide-array.*** *The mature PrP-ORF (excluding N –and C-terminal signal sequences) is divided into 15-mer overlapping peptides, resulting in a grid where in each well the amino-acid sequence has shifted by one amino-acid.*

2. Materials.

2.1 MBP-PrP construction and verification.

1. Difco™ LB Broth (Miller)
2. Difco™ LB Agar (Miller)
3. 100 mg/ml Ampicillin stock (Sigma, -20°C)
4. Amplitaq® Gold DNA polymerase (Applied Biosystems)
5. GeneAmp® PCR buffers (Applied Biosystems)
6. GeneAmp® dNTP blend (Applied Bosystems)
7. Agarose (Merck)
8. TA cloning vector (Invitrogen, -20°C)
9. One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, -20°C)
10. Wizard® *Plus* Minipreps DNA Purification System (Promega)
11. Enzymes *Dra*I and *Eco*RI (New England Biolabs, -20°C)
12. pMAL-c2X expression vector (New England Biolabs, -20°C)
13. Monoclonal PrP-specific antibody (9A2; in house, -20°C, see note 1)
14. Monoclonal MBP-specific antibody (New England Biolabs, -20°C, see note 1)
15. Factor Xa (New England Biolabs)
16. 10% SDS stock (Merck)
17. NuPAGE gel-electroforeses system (Invitrogen):

a. XCell SureLock® Mini-Cell
b. 12% NuPAGE® Novex Bis-Tris Gel (4°C)
c. 20x NuPAGE® MOPS SDS Running Buffer (4°C)
d. NuPAGE® Antioxidant (4°C)
e. MagicMark™ XP Western Protein Standard (4°C)
f. XCell II™ Blot Module CE Mark
g. NuPAGE® Transfer Buffer (20X) (4°C)
h. Ureum sample buffer (4M ureum + 10% β-mercaptoethanol, -20°C)

18. Hybond™-C Extra (Amersham, optimized for protein transfer)
19. 0,05% PBS-Tween tablets (Calbiochem, RT)
20. SYPRO® Orange protein gel stain (Invitrogen, 5000X concentrate in DMSO, 4°C)
21. STORM 840 / Typhoon (GE Healthcare, life sciences)
22. ECF™ substrate for Western blotting (GE Healthcare, -20°C, see note 2)

2.2 MBP-PrP expression and analysis.

1. Difco™ LB Broth (Miller, fresh made & autoclaved prior to use)
2. 100 mg/ml Ampicillin stock (Sigma, -20°C)
3. 2 g/l glucose (Merck)
4. 0,1 M IPTG stock (Sigma, -20°C)
5. E.coli containing pMAL-MBP-PrP (glycerol-stock, -80°C)
6. protease inhibitors (1000x stock) (-20°C, see note 3):
   a. 25 mg/ml Pefabloc-Sc (AEBSF) in dH2O [500mg, Roche]
   b. 700 µg/ml Pepstatine (1 µM) in MeOH [10mg, Roche]
   c. 500 µg/ml Aprotinine (0.15 µM) in dH2O [10mg, Roche]
   d. 500 µg/ml Leupeptine (1µM) in dH2O [25mg, Roche]
   e. 0.5 M EDTA, pH8.0 (Merck, RT)
7. demineralised water (MiliQ)
8. Methanol, analysis grade (Merck)
9. Column-buffer (RT): 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1mM EDTA pH 8.0, 10 mM β-mercaptoethanol (added fresh prior to use).
10. Cuphorn sonicator (Branson)
11. 10% Triton X-100 stock (BDH, RT)
12. amylose slurry (New England Biolabs, supplied pre-swollen in 20% ethanol, 4°C)
13. 5 ml disposable columns (Qiagen)
14. 1 M Maltose stock (Merck, 4°C)
15. 0,5 ml siliconized screw-cap tubes (BioPlastics)

16. NuPAGE gel-electroforeses system (Invitrogen)
17. Hybond™-C Extra (Amersham, optimized for protein transfer)
18. 0,05% PBS-Tween tablets (Calbiochem, RT)
19. SYPRO® Orange protein gel stain (Invitrogen, 5000X concentrate in DMSO, 4°C)
20. 10% non immune goat serum (Zymed laboratories inc.)
21. Monoclonal MBP-specific antibody (New England Biolabs, -20°C, see note 1)
22. STORM 840 / Typhoon Trio (GE Healthcare, life sciences)
23. ECF™ substrate for Western blotting (GE Healthcare, -20°C, see note 2)

2.3 Peptide-array analysis.

Materials needed for synthesis and coupling of 15-mer peptides to form the ovine prion protein peptide-array are to a certain extent described in [13-16], however the exact methodological details are the intellectual property of Pepscan Systems BV, who perform these experiments on a contractual basis. Measurement of coloration of the substrate and subsequent translation to the optical density requires a specialized digital camera and analysis software setup as described by Slootstra *et al*. [15]. The whole procedure, except for subsequent analysis of the final density values, was performed by Pepscan Systems BV.

1. SQ-buffer (4°C): 5% (v/v) horse serum, 5% (v/v) hen albumin, 1% (v/v) Tween 80 in PBS
2. Monoclonal MBP-specific antibody (New England Biolabs, -20°C, see note 1)
3. Rabbit anti-mouse-IgG antibody coupled horse radish peroxidase antibody (Dako, 4°C)
4. 0,05% PBS-Tween (Calbiochem, RT)
5. ABTS peroxidase substrate (Sigma-Aldrich)
6. Hydrogen peroxide (Merck, H_2O_2)
7. CCD camera and image processing system:
    a. Sony CCD Video Camera XC-77RR
    b. Nikon micronikkor 55 mm f/2.8 lens
    c. Camera adaptor (Sony Camera adaptor DC-77RR)
    d. Image processing softwarepackage TIM, v. 3.36 (Difa Measuring Systems, Breda, The Netherlands).

3. Methods.

3.1 MBP-PrP construction and verification.

Polymerase Chain Reaction (PCR) was performed conform standard conditions. Cloning of the PCR-product into the TA-cloning vector and sub-cloning into pMAL-c2X was performed conform the protocols supplied by the manufacturers.

1. In order to obtain the PrP gene suitable for cloning into the pMAL Protein Fusion and Purification System, the mature part of the ovine PrP (ARQ) open reading frame (ORF) was PCR amplified using platinum *Taq* polymerase and PrP specific primers ShBo-F-DraI (5'-GGTGGTTTTAAAAAGCGACCAAAACCTGG-3') and Sh-R-STOP (5'-GGTGGTCT-ATGCCCCCCTTTGGTAATAAGCC-3') using standard 50µl PCR reaction containing: 100ng DNA template, 5µl PCR Buffer (10x), 0.5µl dNTPs (50 mM), 1.0µl of each primer (10mM), sterile water to a total volume of 49 µl and 1 unit of Taq Polymerase.

2. In order to check the integrity and purity of PCR product 5,0µl of the PCR reaction was run on a 1% agarose gel. The PCR produced a single band of appropriate size; therefore the PCR product was directly cloned into the TA-vector (see note 4).

3. The resulting PrP (AA25-233), without its N -and C-terminal signal sequences, was cloned into a general TA-cloning vector using the TA Cloning® Kit, by adding 1,0µl of fresh PCR-product to 50ng of the TA-vector, 1,0µl of 10x ligation buffer, 1,0µl of T4 DNA Ligase (4 Weiss units) and make up to a total volume of 10,0µl with sterile water.

4. After ligation of the PCR-product in the TA-vector, the complete ligation reaction was transformated in the commercial TOP10 chemically competent *E.coli* and the transformated cells spread on LB-agar plates containing 100µg/ml Ampicillin.

5. Single colonies were picked and grown overnight at 37°C while shaking in 2ml LB-broth containing 100µg/ml Ampicillin.

6. Plasmid DNA was isolated using the Wizard® *Plus* Minipreps DNA Purification System for further use.

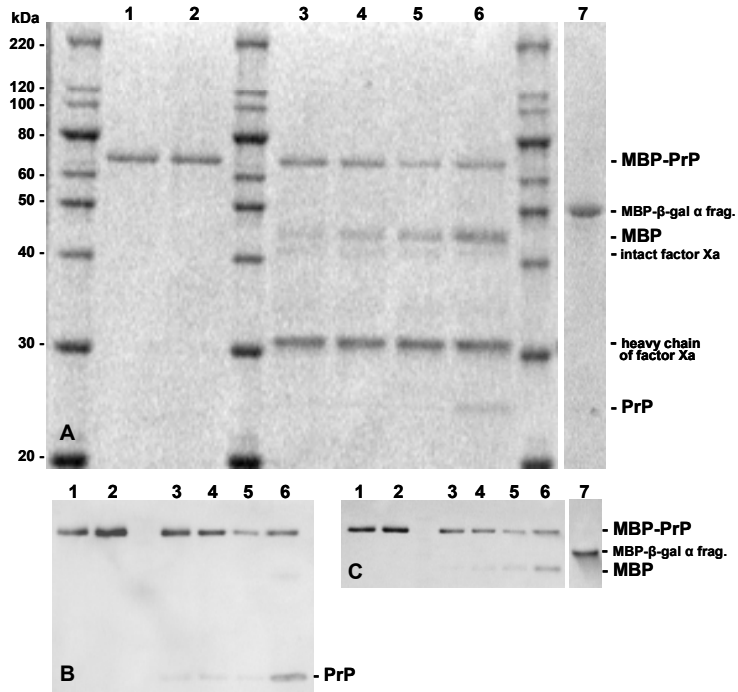
7. Before sub-cloning the mature PrP ORF in the pMAL-c2x expression plasmid, the PrP ORF was sequenced to exclude PCR artefacts.

8. The PrP fragment was subsequently sub-cloned using *Dra*I and *Eco*RI into the pMAL-c2X expression vector, resulting in the maltose binding protein (MBP) fusion to the N-terminus of PrP (MBP-PrP).

In order to assess whether the complete mature part of PrP^C was expressed correctly and whether the fusion protein had accessible folding, MBP-PrP was expressed and analyzed as described in 3.2 below. MBP-PrP was digested with factor Xa as described in the manual of the pMAL Protein Fusion and Purifications System. Expression of maltose binding protein N-terminally fused to PrP (MBP-PrP) revealed a mainly soluble recombinant MBP-PrP of approximately 70 kDa (Fig.2, lanes 1 & 2) and the naked PrP protein could be obtained by digestion with protease Factor Xa, indicating accessible folding (Fig.2, lanes 3-6). After 24 hours approximately 45% of MBP-PrP was digested by factor Xa, however when aided by addition of 0.01% SDS factor Xa completely digested MBP-PrP within 24 hours (data not shown). Furthermore, MBP-PrP is readily detected in Western blot using both a PrP-specific antibody (9A2, Fig.2B) and a MBP specific antibody (Fig.2C). Several monoclonal and polyclonal antibodies with recognition-sites dispersed throughout the PrP protein detected MBP-PrP in Western blot, indicating complete expression of the mature part of sheep PrP^C (data not shown). MBP expressed without additional fusion protein (PrP), which frequently served as negative control in this study, was also of homogeneous quality (Fig 3, lane 7) and of expected size (MBP-β-gal α fragment, 50.8 kDa) which is somewhat larger (as expected) than MBP cleaved from the fusion protein after factor Xa digestion (42.5 kDa).

Figure 2 (following page). ***Analysis of MBP-PrP and MBP expression and MBP-PrP digestion by Factor Xa.*** *Lane 1 contains untreated MBP-PrP, whereas lane 2 contains a mock digestion of MBP-PrP. MBP-PrP was digested with 1% w/w factor Xa and during digestion samples were taken at 2, 4, 7 and 24 hours (lanes 3,4,5 and 6 respectively). All samples were run on SDS-PAGE and the gel was stained with Sypro Orange (total protein stain, panel A) before western blotting and subsequent immunodetection using either a PrP-specific monoclonal antibody (9A2, panel B) or MBP-specific monoclonal antibody (α-MBP, panel C). Expression of MBP, expressed from the pMAL-c2X vector with no insert (MBP-β-gal α fragment), was analyzed by Western Blot using either 9A2 or α-MBP (lane 7, panel A & C respectively). [reproduction of figure 1, published in BMC Biochemistry by Rigter et al. [12]].*

Figure 2.



3.2 MBP-PrP expression and analysis.

Expression and purification by affinity chromatography was performed as described in the manual of the pMAL Protein Fusion and Purifications System (method I), with the following adaptations;

1. The litre of rich broth (LB-broth containing 2g/l glucose and 50 μg/ml Ampicillin) was inoculated with 50ml overnight culture.

2. To improve binding of MBP-PrP by preventing formation of interchain disulfide bonds upon lysis (as suggested in the protocol), 10mM β-mercaptoethanol was added to the column buffer.

3. Before sonication of the cell suspension an inhibitor cocktail (Pefabloc Sc, Pepstatine, Aprotinine, Leupeptine and EDTA) was added and the cell suspension was sonicated 8 times for 15 seconds, resting the cell-extract on ice for 45 seconds between sonication rounds.

4. To ensure optimal lyses of the cells 1% (v/v) Triton X-100 was added to the sonicated cell suspension and incubated for 20 minutes at 4°C, while gently rocking.

5. After lyses the dilution of the crude extract with column buffer was not necessary.

6. A column was prepared using 2ml of 20% amylase slurry and the MBP-PrP fusion protein was eluted after binding with column buffer containing 10mM Maltose in 10 fractions of 250µl.

Quantity and quality of the eluted MBP-PrP was determined by SDS-PAGE as follows.

1. From each collected fraction a 10µl sample was taken and run on a 12% NuPAGE® Novex Bis-Tris Gel together with a bovine serum albumin (BSA) concentration curve samples (2.0, 1.0, 0.5 and 0.25 µg, see note 5).

2. After separation by electroforesis, the gel was taken out of its casing, trimmed and washed in 1x NuPAGE® Transfer Buffer for 10 minutes to remove excess unbound SDS from the gel (see note 6).

3. The gel was then transferred to fresh 1x NuPAGE® Transfer Buffer containing 1:5000 diluted Sypro® Orange and incubated for 1 hour (see note 7).

4. The gel was then rinsed in 1x NuPAGE® Transfer Buffer and scanned using the STORM 840 using standard settings (see note 8).

5. After scanning the gel was blotted for 1 hour at constant voltage of 30V onto nitrocellulose (Hybond™-C Extra) using the XCell II™ Blot Module CE Mark and NuPAGE® Transfer Buffer (see note 9).

6. The resulting blot was transferred to a 50ml falcon tube containing 40ml blocking buffer (5% milk protein in PBS) and incubated for 1 hour at room temperature while gently shaking. Alternatively the blot can be incubated overnight at 4°C.

7. After blocking the blot was rinsed once in 40ml of 0,05% PBS-Tween.

8. The blot was then incubated in 5ml PBS-Tween containing 1% non immune goat serum (NGS) and either 1:1500 diluted MBP-specific antibody or 1:1000 diluted PrP-specific antibody 9A2 as primary antibody, for 1 hour at room temperature while gently shaking.

9. After incubating with the primary antibody, the blot was rinsed once in 40ml of PBS-Tween (0,05%).

10. After rinsing the blot was washed 2 times in 25ml 0,05% PBS-Tween for 10 minutes while gently shaking.

11. The blot was then incubated in 5ml 0,05% PBS-Tween containing 1% NGS and 1:2000 diluted goat-anti-mouse-IgG coupled with alkaline phosfatase as the secondary antibody, for 1 hour at room temprature while gently shaking.

12. After incubating with the secondary antibody, the blot was rinsed once in 40ml of 0,05% PBS-Tween.

13. After rinsing the blot was washed 2 times in 25ml 0,05% PBS-Tween for 10 minutes while gently shaking.

14. The 0,05% PBS-Tween of the final wash is discarded and 50ml of fresh 0,05% PBS-Tween was added for transport and storage at 4°C (see note 10).

15. The surface area of the blot was determined and 24 µl/cm^2 ECF was pipetted directly onto the glass plate of the STORM 840.

16. The blot was taken out of the 0,05% PBS-Tween in which it was stored, and gently dried before placing the blot onto the ECF, making sure the ECF spreads underneath the whole blot (see note 11).

17. The blot was immediately scanned using the STORM 840 scanning software and standard settings (see note 12) for ECF and the scan was repeated after 5, 10, 15 and if necessary 20 minutes (see note 13) of the start of the initial scan.

18. Using the ImageQuant software supplied by the manufacturer, the band density was determined for further analysis.

3.3 Peptide-array analysis.

Synthesis of complete sets of overlapping 15-mer peptides (Fig.1) were carried out on grafted plastic surfaces, covering the ovine PrP amino acid sequence of mature PrP^C (residues 25-234 of ovine and 25-242 of bovine PrP) [17]. The plastic surface consisted of 455, 3µl wells on a credit-card size plastic (minicard) carrier. In each well the peptides were coupled to the grafted plastic surface in two ways: either with direct step-by-step coupling of amino acids or by coupling complete 15-mer peptides at their C-terminus [13-16] and subsequent ELISA analyses were performed. The established procedures for peptide-array analysis are used to determine antibody recognition domains (Fig.3A), however when using the prion protein peptide-array to determine interaction domains of PrP, direct detection with α-PrP antibodies can possibly recognize peptides that did not bind PrP and thus frustrate the results. However by incubating MBP-PrP as the antigen that binds to the peptide, detection of the fusion protein is possible using α-MBP antibody, followed by rabbit α-mouse-HRP (Fig.3B), preventing frustration of the results due to cross-reaction between the primary antibody and the ovine prion protein derived peptides. The procedure described below is not extensive; however points 2-4 entail the necessary adaptation of the standard peptide-array analysis protocol, which allows for elucidation of the amino acid sequences involved in protein-protein interaction.

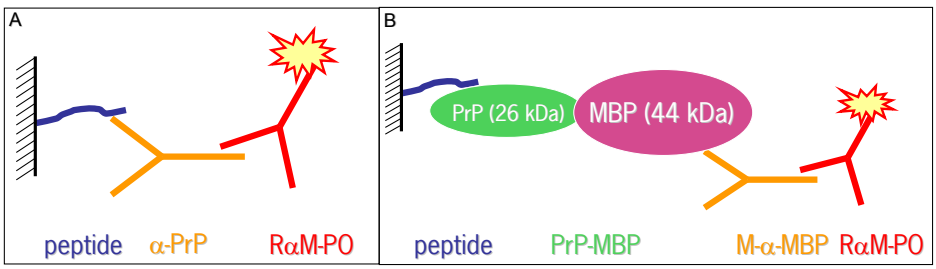


Figure 3. ***Elisa based detection principle of peptide-array analysis.*** ***(A)*** *Standard detection setup for a peptide-array in order to determine antibody epitope and* ***(B)*** *Alternative setup used to determine peptide-protein interaction in this study.*

1. Support-coupled peptides were pre-coated with SQ-buffer for 1 hr at 37°C to block non-specific absorption of antibodies.

2. MBP-PrP was diluted in SQ-buffer (standard buffer used by Pepscan B.V.) to a concentration resulting in a sufficient relative density value, allowing differentiation between peaks (Fig. 4, column graph), without affecting the background and preventing the relative density value reaching its plateau. In the case of MBP-PrP a concentration of 7,5-10 µg/ml was optimal (see note 14).

3. MBP-PrP was incubated overnight at 4°C as an antigen on the peptide-array minicard.

4. The peptide-array minicard was washed three times in 0,05% PBS-Tween and all buffer was removed from the wells.

5. The peptide-array minicard was incubated with the primary MBP-specific antibody diluted 1:2000 in SQ-buffer for 1hour at 25°C.

6. The peptide-array minicard was washed three times in 0,05% PBS-Tween and the buffer was removed from all the wells.

7. The peptide-array minicard was incubated with the secondary rabbit anti-mouse-IgG antibody coupled horse radish peroxidase antibody diluted 1:1000 in SQ-buffer for 1hour at 25°C.

8. The peptide-array minicard was washed three times in PBS-Tween (0,05%) and the buffer was removed from all the wells.

9. After washing the peroxidise substrate ABTS and 2µl/ml 3% H_2O_2 was added and incubated for 1 hour at room temperature.

10. The optical density of the signals in the separate wells we measured using a CCD camera and an image processing system as described by Slootstra *et al.* [15].

11. The measured density values were further analysed as follows:

12. The background was determined by calculating the mean value of 20 peptides with low density values of which at least 5 peptides were in consecutive order. The relative density value (r.d.v.) was calculated by dividing the optical density value (o.d.v.) by the background and binding was considered relevant when at least 3 consecutive peptides showed binding values of at least 3 times the background.

Interaction between the individual PrP sequences (peptides) and MBP-PrP was sufficient for immunodetection, resulting in a reproducible binding pattern (Fig.4, column graph). This binding pattern, expressed in relative density values was characterized by two distinct high binding areas (peptides 35-102 and 134-102 respectively) as well as some lower binding areas. Analysis of the correlating peptide sequences revealed that these areas usually were characterized by consensus sequences which suggested the existence of distinct interaction domains for the mature part of PrP^C (for detailed analysis see [12]). To further assess the extent of the specificity of the binding pattern found, MBP-PrP was also tested against a bovine PrP peptide-array. This yielded a similar binding pattern comparable to the results with ovine PrP peptide array but with some slight differences, which can be correlated to differences in amino acid differences between the ovine –and bovine prion protein [12] (data not shown).

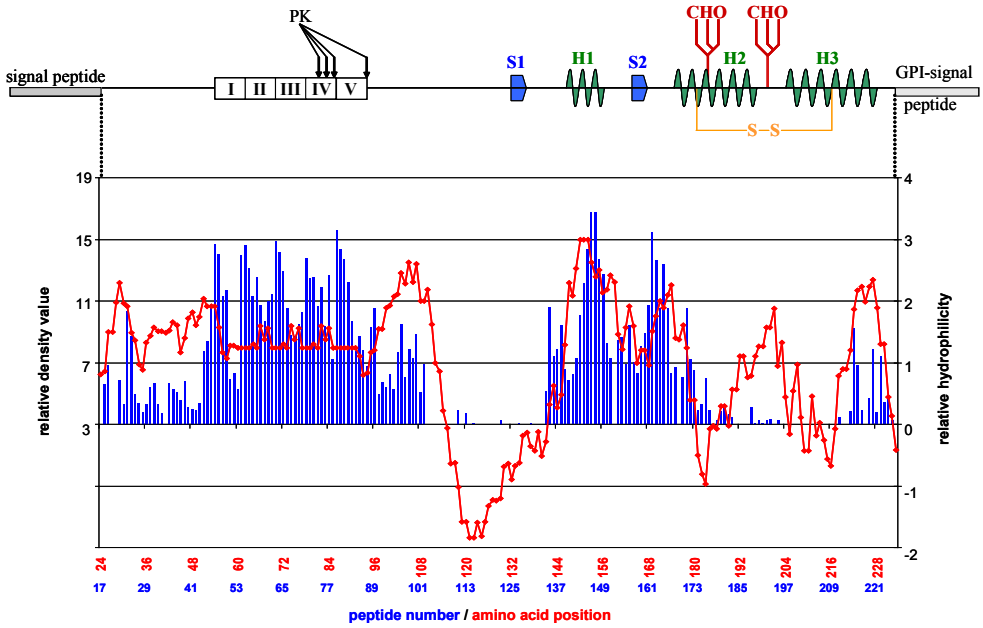


Figure 4. ***Overview of PrP^C secondary structures and antibody epitopes versus peptide-array binding pattern and Kyte-Doolittle hydrophilicity plot.*** *Schematic representation of PrP^C showing signal sequences, β-sheets (S1, S2), α-helices (H1, H2, H3), disulfide bridge site (S-S) and glycosylation sites (CHO). The sequence of PrP is lined up with both the Kyte-Doolittle hydrophilicity plot (line graph; negative = hydrophobic and positive = hydrophilic) and the relative binding pattern found (column graph) with the ovine prion peptide-array. [adaptation of figure 5, published in BMC Biochemistry by Rigter et al. [12]]*

To find a correlation with structural properties, the relative binding pattern of MBP PrP on the peptide array was compared to the Kyte-Doolittle hydrophilicity plot of mature PrP^C (Fig.4, line graph) revealing a high correlation between hydrophilic (exposed) regions of PrP^C and the observed binding pattern regions.

4. Notes.

1. Storage of the antibody stocks is done best at -20°C. Stocks are best divided in aliquots before storage. When an aliquot is taken into use it is best to store this aliquot at 4°C to prevent antibody degradation by repeated thaw-freezing until finished.

2. ECF is best stored as aliquots of 1ml (about two blots) to minimize decline of the substrate as a result of repeated thaw-freezing.

3. For ease of use it is best to aliquot the protease inhibitors (except EDTA) and store at -20°C, for prolonged storage. The aliquot in use can be stored at 4°C until finished.

4. When the PCR does not produce a single band it is pertinent to run the whole PCR reaction on an agarose gel and excise the band that correlates to the specific PCR product wanted. Extract the DNA from the gel size using a commercially available kit. However, the amount of PCR product recovered is usually low and if possible it is advised to optimize the PCR reaction in order to get a single band. Then the procedure described can be followed, with greater chance of success.

5. After staining with Sypro Orange and visualisation using the STORM 840, the ImageQuant software supplied by the manufacturer can be used to determine the (relative) density of the BSA bands, these densities ('volumes') can be extrapolated against the concentration of BSA loaded in the lane, resulting in a (linear) concentration curve. From this curve the concentration of the protein present in the fractions can be estimated using the determined band volumes for these fractions.

6. Wash at least for 10 minutes, but no more than 30 minutes. Sypro Orange binds to protein bound SDS. Washing helps eliminate background colouring; however, prolonged washing diminishes the protein staining.

7. Sypro Orange is light sensitive; therefore incubating in a closed light-impermeable container is advised for optimal results.

8. Sypro Orange has an excitation max of 300,470 nm, and an emission max of 570 nm, use Blue fluorescence mode (automatically coupled to emission filter) on STORM 840.

9. Correct stacking of the gel, nitrocellulose and blot-pads is described in the information provided by the manufacturer.

10. The blot can be stored at 4°C in PBS-Tween for several weeks without loss of signal. However storage may cause the signal to come up later compared to direct measuring using ECF and the STORM 840.

11. When placing the blot onto the ECF, make sure no bubbles are trapped underneath the blot. It is easier to add extra ECF when necessary afterwards, when to much ECF is present this will flow out from underneath the blot and will create smearing during scanning.

12. ECF has an excitation max of 440 nm and an emission max of 560 nm, use the Blue fluorescence mode (automatically coupled to emission filter) on STORM 840.

13. Scanning at different time-intervals allows for choosing the scan in which good signal is present, but in which the highest signal has not yet reached its plateau. This ensures that the subsequent density measurements of the bands can be compared in further analysis.

14. During the experiments we discovered that soluble MBP-PrP protein interacts with the peptides on the peptide-array. Aggregation of MBP-PrP resulted in disappearing of signal, therefore slight differences in optimal MBP-PrP concentration are likely due to differences in solubility between different batches of MBP-PrP used.

Acknowledgements.

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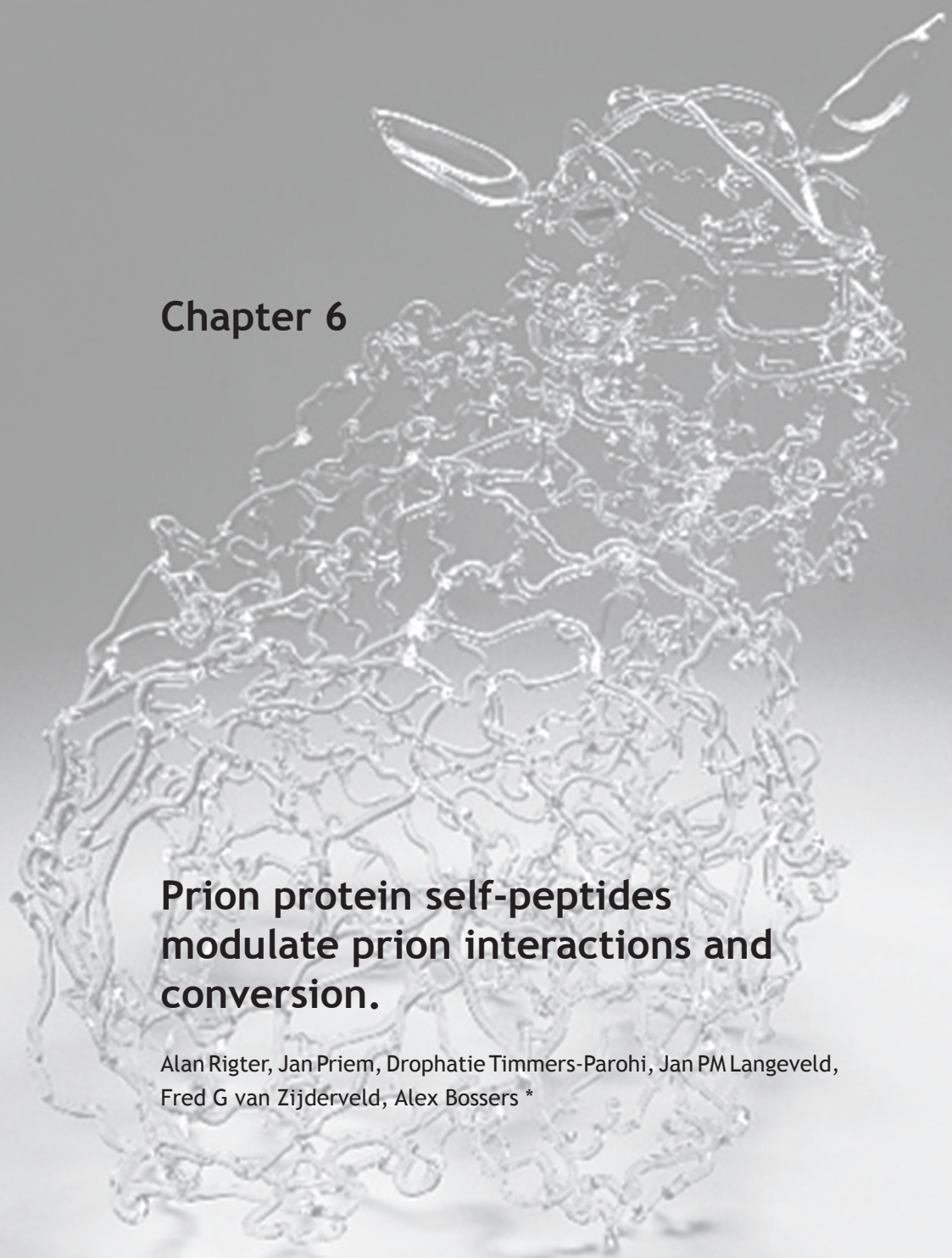
References.

1. DeArmond, S.J. and S.B. Prusiner, *Perspectives on prion biology, prion disease pathogenesis, and pharmacologic approaches to treatment.* Clin Lab Med, 2003. 23(1): p. 1-41.
2. Atarashi, R., et al., *Prion strain-dependent differences in conversion of mutant prion proteins in cell culture.* J Virol, 2006. 80(16): p. 7854-62.
3. Bossers, A., *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie.* 1999, University of Utrecht.
4. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.
5. Bossers, A., et al., *In vitro conversion of normal prion protein into pathologic isoforms.* Clin Lab Med, 2003. 23(1): p. 227-47.
6. Bossers, A., et al., *PrP genotype contributes to determining survival times of sheep with natural scrapie.* J Gen Virol, 1996. 77 (Pt 10): p. 2669-73.
7. Kocisko, D.A., et al., *Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier.* Proc Natl Acad Sci U S A, 1995. 92(9): p. 3923-7.
8. Raymond, G.J., et al., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo J, 2000. 19(17): p. 4425-30.
9. Raymond, G.J., et al., *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans.* Nature, 1997. 388(6639): p. 285-8.
10. Sabuncu, E., et al., *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol, 2003. 77(4): p. 2696-700.
11. Rigter, A. and A. Bossers, *Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion.* J Gen Virol, 2005. 86(Pt 9): p. 2627-34.
12. Rigter, A., et al., *Mapping of possible prion protein self-interaction domains using peptide arrays.* BMC Biochem, 2007. 8: p. 6.
13. Geysen, H.M., R.H. Meloen, and S.J. Barteling, *Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid.* Proc Natl Acad Sci U S A, 1984. 81(13): p. 3998-4002.
14. Piccardo, P., et al., *An antibody raised against a conserved sequence of the prion protein recognizes pathological isoforms in human and animal prion diseases, including Creutzfeldt-Jakob disease and bovine spongiform encephalopathy.* Am J Pathol, 1998. 152(6): p. 1415-20.
15. Slootstra, J.W., et al., *Structural aspects of antibody-antigen interaction revealed through small random peptide libraries.* Mol Divers, 1996. 1(2): p. 87-96.

16. Timmerman, P., et al., *Mapping of a discontinuous and highly conformational binding site on follicle stimulating hormone subunit-beta (FSH-beta) using domain Scan and Matrix Scan technology.* Mol Divers, 2004. 8(2): p. 61-77.

17. Goldmann, W., et al., *Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon.* J Gen Virol, 1991. 72 (Pt 1): p. 201-4.



Chapter 6

Prion protein self-peptides modulate prion interactions and conversion.

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Prion protein self-peptides modulate prion interactions and conversion.

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Abstract.

Background. Molecular mechanisms underlying prion agent replication, converting host-encoded cellular prion protein (PrP^{C}) into the scrapie associated isoform (PrP^{Sc}), are poorly understood. Selective self-interaction between PrP molecules forms a basis underlying the observed differences of the PrP^{C} into PrP^{Sc} conversion process (agent replication). The importance of previously peptide-scanning mapped ovine PrP self-interaction domains on this conversion was investigated by studying the ability of six of these ovine PrP based peptides to modulate two processes; PrP self-interaction and conversion.

Results. Three peptides (octarepeat, binding domain 2 -and C-terminal) were capable of inhibiting self-interaction of PrP in a solid-phase PrP peptide array. Three peptides (N-terminal, binding domain 2, and amyloidogenic motif) modulated prion conversion when added before or after initiation of the prion protein misfolding cyclic amplification (PMCA) reaction using brain homogenates. The C-terminal peptides (core region and C-terminal) only affected conversion (increased PrP^{res} formation) when added before mixing PrP^{C} and PrP^{Sc}, whereas the octarepeat peptide only affected conversion when added after this mixing.

Conclusions. This study identified the putative PrP core binding domain that facilitates the PrP^{C}-PrP^{Sc} interaction (not conversion), corroborating evidence that the region of PrP containing this domain is important in the species-barrier and/or scrapie susceptibility. The octarepeats can be involved in PrP^{C}-PrP^{Sc} stabilization, whereas the N-terminal glycosaminoglycan binding motif and the amyloidogenic motif indirectly affected conversion. Binding domain 2 and the C-terminal domain are directly implicated in PrP^{C} self-interaction during the conversion process and may prove to be prime targets in new therapeutic strategy development, potentially retaining PrP^{C} function. These results emphasize the importance of probable PrP^{C}-PrP^{C} and required PrP^{C}-PrP^{Sc} interactions during PrP conversion. All interactions are probably part of the complex process in which polymorphisms and species barriers affect TSE transmission and susceptibility.

Background.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders characterized by accumulation of the pathological isoform of prion protein mainly in tissues of the central nervous system. Formation of this pathological isoform is a posttranslational process and involves refolding (conversion) of the host-encoded prion protein (PrP^{C}) into a pathological isoform partially protease resistant PrP^{Sc} (derived from scrapie) or PrP^{res} (PK-resistant PrP) [1]. The molecular mechanisms involved in PrP^{C} to PrP^{Sc} conversion are poorly understood, but polymorphisms in both PrP isoforms have been shown to be of importance in both interspecies and intraspecies transmissibilities [2]. The formation of PrP^{Sc} aggregates probably requires self-interactions of PrP^{C} molecules as well as with PrP^{Sc} [3, 4]. Thus binding and conformational changes are essential events in this conversion process. Cell-free conversion of PrP^{C} provides a valuable *in vitro* model in which relative amounts of produced PrP^{res} reflect important biological aspects of TSEs at the molecular level [5, 6]. A recent and very sensitive *in vitro* conversion system is the protein misfolding cyclic amplification (PMCA) assay [7-10], which has been shown to amplify minute amounts of PrP^{Sc} from a variety of sources including sheep scrapie [10]. The effects of single polymorphisms and species-barriers in PrP^{C} or PrP^{Sc} on PrP conversion can largely explain differences in susceptibility -and transmissibility in sheep scrapie [5, 11-13]. Even though these polymorphisms are involved in modulation of disease development they do not seem to affect the initial binding of PrP^{C} to PrP^{Sc} [14] and do not seem to directly modulate PrP^{C}-PrP^{Sc} binding. Furthermore, in a recent peptide-array mapping study of ovine PrP^{C} we concluded that these polymorphisms are not part of the identified PrP binding domains likely to be involved in PrP self-interaction [15]. However, this does not exclude these polymorphisms from posing indirect effects on binding behavior of PrP^{C} to PrP^{Sc} and other possible chaperoning molecules. In that peptide-array binding study we unequivocally demonstrated that ovine PrP binds with PrP derived (self) amino acid sequences (sequence specific) separate from the polymorphic scrapie susceptibility determinants [15]. It remains to be elucidated whether the determined amino acid sequences play a role prior or during conversion in the self-interaction of PrP^{C} molecules and/or in the interactions of PrP^{C} with PrP^{Sc}. Simultaneously, whether these amino acid sequences play a role in the processes underlying PrP conversion needs to be elucidated. In the current study we selected several ovine PrP sequence derived synthetic peptides to study not only their capacity to affect PrP binding to a solid-phase (PrP) peptide-array but also their potential modulating effect on PrP^{C} to PrP^{Sc} conversion.

Results.

Previously we determined that recombinant ovine PrP yielded a reproducible sequence specific binding pattern with amino acid sequences using a solid-phase array of overlapping 15-mer peptides encompassing the complete ovine –or bovine amino acid sequence (peptide-array). Roughly this pattern breaks down into two high binding areas containing two–and three consensus domains respectively, combined with some lower binding domains (Figure 1). Based on the interaction domains extrapolated from this binding pattern as well as properties reported in literature, the following six ovine PrP regions were selected for peptide blocking studies. The sequences of these peptides represented structural properties of PrP as explained hereafter (summarized in table 1 and mapped in figure 1): **Peptide NTG**, spanning the amino acids (AAs) at the N-terminal part of the mature PrP^{C}, including the glycosaminoglycan (GAG) binding motif KKRPK [16] and binding domain 1 [27-RPKPGGG-33] (ovine numbering used throughout, [15] and this study); **Peptide OR,** spanning the octarepeat AA motif [QPHGGGWG, AA 54-94] of the N-terminal region (PrP self-interaction was mapped to AA motif P(H)GG [15]), which is probably involved in a range of interactions [17-31] of which metal-binding is the best characterized; **Peptide TD2**, which overlaps the limiting region containing strain and species dependant variable sites for proteinase K trimming of PrP^{Sc} [32, 33] and spanning binding domain 2 ([102-WNK-104], ovine numbering used throughout) of the first high binding area [15]; **Peptide AM**, which includes the amyloidogenic motif (AGAAAAGA) of PrP that did not exhibit any binding in the peptide-array [15]; **Peptide CO**, encompassing amino acids of the core region of PrP spanning from the first β-sheet onto the first α-helix. The peptide includes binding domain 3 [140-PLIHFGNDYE-149] and is immediately adjacent to binding domain 4^{a} of the second high binding area [15]; These domains are also important in PrP^{Sc} conformation-specific immuno-precipitation [34-40]; and **peptide CT**, spanning the C-terminal AA's covering part of the third helix, partially covering low binding domain 6 [192-TTTTKGENFT-202] and almost identical to a peptide capable of inhibiting cell-free conversion [41].

PrP peptide inhibition of PrP self-binding to peptide-array.

First these six peptides were tested for their capability to inhibit PrP binding to the PrP based peptide-array containing 242 peptides (15-mer) overlapping each other by increments of 1 AA, covering the complete ovine PrP amino acid sequence (results summarized in Table 1).

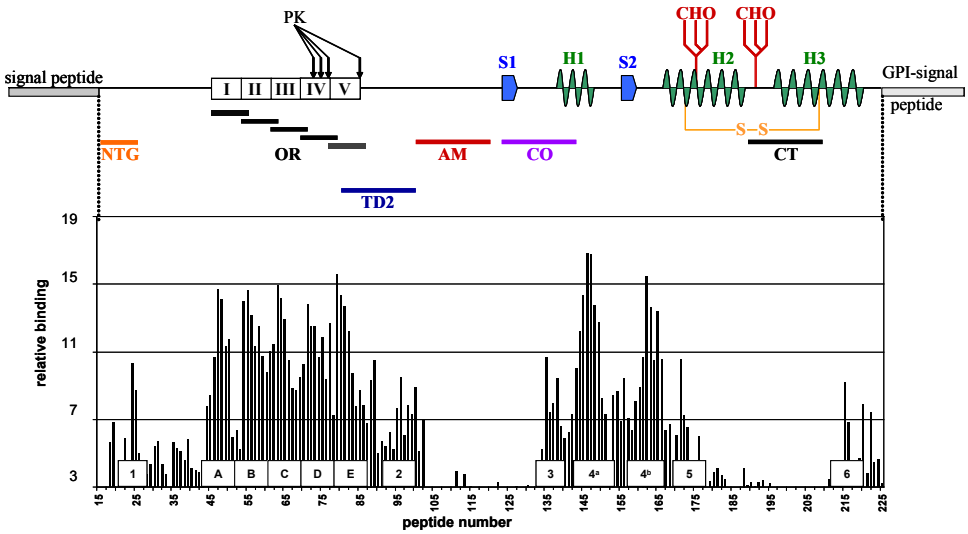


Figure 1. ***PrP^C secondary structures and relative peptide positions versus peptide-array binding pattern and binding domain positions.*** *Schematic representation of PrP^C showing signal sequences, β-sheets (S1, S2), α-helices (H1, H2, H3), disulfide bridge (S-S), glycosylation sites (CHO) and the relative positions of the peptides used in this study. The bar graph represents the previously determined peptide-array binding pattern [15] and the relative positions of the determined interaction domains: 1 [27-RPKPGGG-33], A-E octarepeat motif [PxGG], 2 [102-WNK-104], 3 [140-PLIHFGNDY-148], 4^a [152-YYR-154], 4^b [165-YYR-167], 5 [177-NFV-179] and 6 [225-SQAY-228]. All numbering used is for sheep PrP.*

Pre-incubation of PrP with peptide CO did not result in blocking of the binding pattern of PrP on the peptide-array (Figure 2a), whereas peptides NTG and AM only moderately blocked the binding pattern of PrP (Figure 2b). Peptide NTG seems to diminish binding throughout the binding pattern (Figure 2b, blue line), whilst peptide AM mainly affects binding with the peptides derived from the N-terminal part of mature PrP (Figure 2b, green line). Maximum blocking throughout the PrP binding pattern occurred with peptides OR (Figure 2c, blue line), TD2 (Figure 2c, green line) and CT (Figure 2c, orange line), which all block equally throughout the PrP binding pattern. However, in contrast to blocking studies performed with antibodies [15], blocking was not absolute over the whole region of the PrP peptide-array binding pattern. Inhibition by the aforementioned peptides was dose-dependant, with maximum blocking only occurring when peptides

were added at high molecular ratios to PrP. Pre-incubation of PrP with (at least) 400 times molar excess of peptide OR or (at least) 200 times molar excess of peptides TD2 and CT was necessary to obtain maximum blocking of the PrP binding pattern on the peptide-array.

Table 1. ***Peptide information and peptide-array blocking results.***

peptide	amino acid sequence	position[1]	block[2]	equiv.[3]
NTG	KKRPKPGGGWNT	25-36	+/-	403
OR	GQPHGGGWGQ	61-95	++	396
TD2	GGGGWGQGGSHSQWNKPSK	89-107	++	198
AM	KTNMKHVAGAAAAGA	109-123	+/-	502
CO	LGSAMSRLPLIHFGNDYEDR	133-151	no	499*
CT	GENFTETDIKIMERVVEQMC	198-217	++	198

[1] position of the amino acid sequence in mature ovine PrP^{C}

[2] effect of pre-incubation with peptide on binding of PrP to the peptide-array
(-- no effect, +/- moderate blocking, ++ blocking)

[3] minimal amount of molar excess of peptide needed to affect PrP binding
(highest amount of excess peptide tested)*

The capability of the N-terminal peptide NTG to moderately block binding of PrP to the peptide-array and also moderately modulated PrP^{res} formation in the supplemented –and pre-incubated PMCA assay (described below) necessitates re-evaluation of the previously determined consensus domain [33-GWNTG-37] [15]. Instead of this common consensus domain two binding domains seem present in the N-terminus. Binding motifs determined by motif-grafted antibodies [42] suggests that the domain of interest is [27-RPKPGGG-33], which encompasses most of the proposed glycosaminoglycan binding motif [25-KKRPK-29] [16].

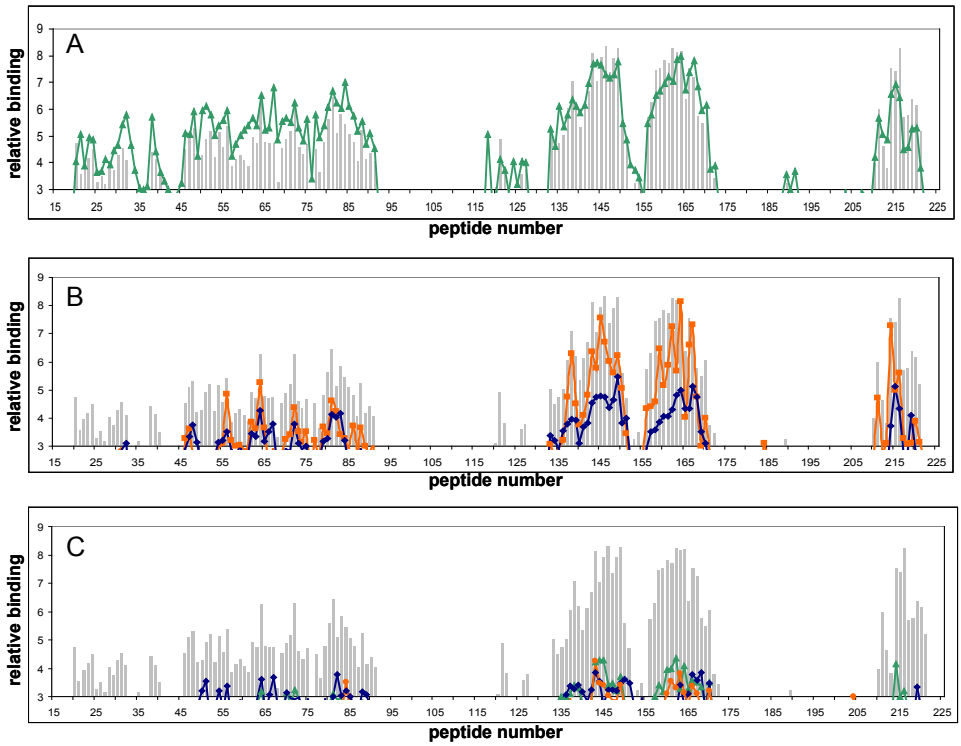


Figure 2. ***Effects of peptides on PrP to peptide-array binding pattern.*** *No effect (A) on the peptide-array binding pattern and intensities was observed when PrP was pre-incubated with peptide CO (green line) compared to non-peptide pre-incubated PrP binding (grey bars). Only moderate inhibition of the binding pattern (B) was observed when PrP was pre-incubated with either peptide NTG (blue line) or peptide AM (orange line), whereas almost complete inhibition was observed when PrP was pre-incubated with either peptide OR (blue line), TD2 (green line) or CT (orange line). Minimal peptide concentration needed for blocking was determined (Table 1).*

These results confirm the importance of the previously mapped domains [15] located within the first high binding area [PHGG] (octarepeat) and [102-WNK-104], as well as the importance of the C-terminal low binding domain [192-TTTTKGENFT-202] in PrP self-interaction**.** To a lesser extent the involvement of the N-terminal glycosaminoglycan binding motif contained within domain [27-RPKPGGG-33] and amyloidogenic motif [116-AGAAAAGA-123] in interaction is confirmed. Interestingly peptide CO, encompassing the previously mapped domain [140-PLIHFGNDYE-149] did not influence binding of PrP to the peptide-array.

PrP peptides modulation of PrPres formation in the PMCA-assay.

The peptides analyzed in the prion protein peptide-array were also studied for their modulating capacity in the sheep PrP protein misfolding cyclic amplification (PMCA) assay [9, 10, 43] using sheep brain homogenates from confirmed scrapie-positive and scrapie-negative sheep in one round of sonication cycles. To test the influence of the peptides on conversion, peptides were either added after combining the scrapie positive –and negative brain homogenates (peptide supplemented PMCA) or alternatively peptide was added first to the scrapie-negative brain homogenate before addition of the scrapie-positive material (peptide pre-incubated PMCA). This allowed us to assess if the effect of the peptides on conversion was dependant on the first rapid interaction between PrP^{C} and PrP^{Sc} [14] or not. Peptide was added in several molar ratios, relative to the calculated total amount of PrP^{C} present in the reaction. PrP^{Sc} specific proteinase-K (PK) resistant fragments were quantified by Western blotting.

Peptide supplemented PMCA: Addition of peptide after mixing scrapie positive -and negative brain homogenates resulted in a dose dependant increase of PK resistant PrP (PrP^{res}) after sonication for four [NTG, OR, TD2 and AM] of the six peptides tested (results summarized in Table 2). In general the amount of newly formed PrP^{res} roughly ranged between 2-fold (standard reaction) up to 8-fold as compared to the input amount of PrP^{Sc}. Addition of a large molar excess of peptide NTG (Figure 3a) resulted in a significant increase in PrP^{res} formation in a dose dependant manner at molar excesses of 5.000 ($p=0.0161$) and 25.000 ($p=0.0007$). Addition of peptide OR (Figure 3b) resulted in the largest increase of PrP^{res}. However, in contrast to the other peptides the effect was inversely dose dependant and significant at molar excesses of 500 ($p<0.0001$) and 1000 $p=0.0001$). Molar excesses below 500 of peptide OR did not result in significant increase of PrP^{res} as with higher molar excess of this peptide. Addition of peptide TD2 (Figure 3c) resulted in a slight to moderate increase in PrP^{res} formation. PrP^{res} increase was significant only at a molar excess of 25.000 ($p=0.0027$). Addition of peptide AM (Figure 3d) resulted in a moderate and significant increase of PrP^{res}, but only when peptide was added at a molar excess of 5.000 ($p=0.0012$). Addition of either peptide CO (Figure 3e) or CT (Figure 3f) did not significantly influence PrP^{res} formation at the tested molar excesses. However it does seem that these peptides slightly inhibit PrP^{res} formation at molar excesses of 1000 and 5000 respectively.

Peptide pre-incubated PMCA: Pre-incubating scrapie-negative brain homogenate with peptide before initiating conversion with scrapie-positive brain homogenate, surprisingly resulted in increased formation of proteinase-K resistant PrP (PrP^{res}) after sonication

Table 2. ***Peptide modulation of supplemented –and pre-incubated PMCA assay.***

		complemented PMCA-assay				pre-incubated PMCA-assay			
peptide	**m.e.[1]**	**median**	**s.e.m.**	**n[2]**	**sign.[3]**	**median**	**s.e.m.**	**n[2]**	**sign.[3]**
NTG	1000	2.07	0.24	3		4.15	0.60	5	
	5000	3.56	0.73	3	*	5.91	0.76	5	**
	25000	4.68	1.26	3	**	4.13	0.63	5	
OR	500	6.26	1.27	4	***	[§]*n.t.*			
	1000	4.61	0.87	5	***	3.85	0.54	4	
	5000	2.74	0.68	4		4.44	0.85	4	
	25000	2.57	0.33	2		3.99	1.19	4	
TD2	1000	3.71	0.73	8	n.q.	1.73	0.35	4	
	5000	3.00	0.93	8		3.15	0.33	4	**
	25000	4.63	0.62	8	**	2.91	0.34	4	*
AM	1000	1.92	0.27	3		4.36	0.28	7	
	5000	4.03	0.34	3	**	5.38	0.76	7	*
	25000	2.36	0.46	3		5.63	0.50	7	**
CO	1000	1.56	0.02	2		8.59	0.46	5	***
	5000	2.02	0.58	2		4.95	1.43	5	
	25000	1.97	0.22	2		6.09	1.53	5	*
CT	1000	1.80	0.21	2		6.21	0.39	5	**
	5000	1.41	0.29	2		5.23	1.76	5	
	25000	2.85	1.12	2		4.77	0.92	5	

[1] amount of molar excess of peptide compared to PrP^C tested in the PMCA assay

[2] numbers of independent measurements performed

*[3] comparison of conversion ratios of the peptide supplemented –or pre-incubated PMCA and their corresponding standards using the unpaired Student's t-test (p-values < 0.05 are considered statistically different). Significantly different values are marked; p-values between 0.05 and 0.001 with *, p-values between 0.001 and 0.0001 with ** and p-values ≤ 0.0001 with ***. The p-value of a comparison that was not quite significantly different is marked with n.q.*

[§]n.t. = not tested

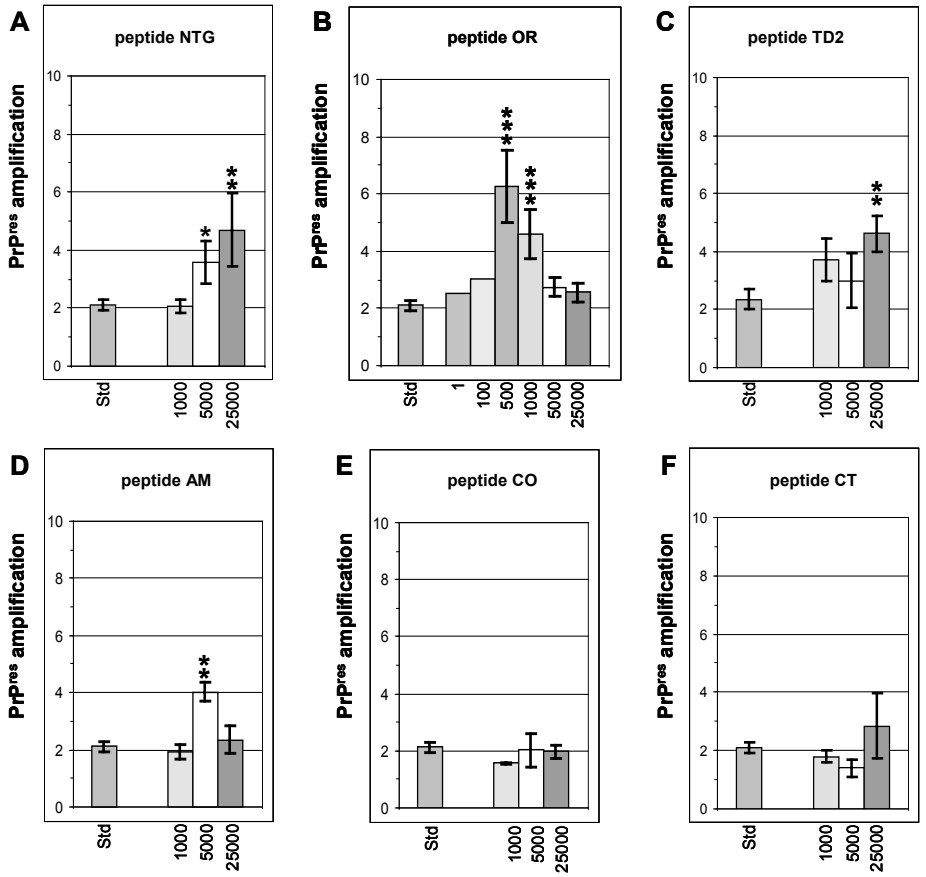


Figure 3. ***Effects of peptide supplementation on PrP^{res} formation in ovine PMCA.*** *Bar graphs depicting PrP^{res} formation of a standard PMCA reaction compared to a PMCA reaction supplemented with different molar excess amounts of peptide NTG (A), peptide OR (B), peptide TD2 (C), peptide AM (D), peptide CO (E) and peptide CT (F). For each peptide its specific corresponding standard is depicted (grey bar). In order to determine the optimal molar excess of octarepeat peptide OR in the supplemented PMCA, a single test with peptide OR added at molar excess 1, 10, 100 and 250 was performed. The amounts of PrP^{res} in these reactions were comparable to the standard. As an example results at molar excess 1 and 100 are depicted in graph B. The number of independent measurements (n), the median and s.e.m. for each peptide and molar excess are summarized in Table 2. An unpaired Student's t-test was performed to determine whether PrP^{res} formation with peptide was significantly different to the corresponding standard PrP^{res} formation. P-values are listed in Table 2 and significant differences are marked; p-values between 0.05 and 0.001 with *, p-values between 0.001 and 0.0001 with ** and p-values ≤ 0.0001 with ***.*

for five [NTG, TD2, AM, CO and CT] of the six peptides tested (results summarized in Table 2) generally in a dose dependant manner. However, compared to the peptide supplemented PMCA assay results differed for several of the peptides. Pre-incubation with peptide NTG (Figure 4a) resulted in a slight but significant increase of PrP^{res} at the optimal molar excess of 5.000 ($p=0.0076$). In contrast to peptide supplemented PMCA reactions, pre-incubation with peptide OR (Figure 4b) did not significantly affect PrP^{res} formation at any of the molar excesses tested. Pre-incubation with peptide TD2 (Figure 4c) induced a moderate but significant increase of PrP^{res} at molar excesses 5.000 ($p=0.0048$) and 25.000 ($p=0.0113$), with an apparent optimum at 5.000. Whereas peptide AM induced amplification of PrP^{res} optimally at molar excess 5.000 in the supplemented PMCA assay, pre-incubation with this peptide (Figure 4d) resulted in a slight but significant dose dependant increase in PrP^{res} at molar excesses 5.000 ($p=0.0362$) and 25.000 ($p=0.0044$). Pre-incubation peptide CO (Figure 4e) resulted in a significant moderate or slight increase in PrP^{res} at molar excesses 1.000 ($p<0.0001$) and 25.000 ($p=0.0496$). However, increase of PrP^{res} at molar excess of 1.000 was larger and unmistakably more significant than at the higher molar excess, suggesting an inverse dose dependant increase of peptide induced PrP^{res} formation. Finally, pre-incubation of SNH with peptide CT (Figure 4f), also resulted in a significant inverse dose dependant increase of PrP^{res} at the molar excess of 1.000 ($p=0.0006$).

PMCA assay negative controls.

Even though each PMCA assay setup revealed at least one PrP specific peptide incapable of modulating PrP^{res} formation, additional negative controls were performed. To rule out the possibility that factors other than PrP sequence specificity could be responsible for the observed results, the isoelectric point, net charge and average hydrophilicity were determined for each peptide (data not shown). Comparison revealed no clear correlation between these non-sequential features and the observed effects in the PMCA assay. Therefore the following additional negative controls were performed; In order to determine whether addition of just a random peptide is sufficient for modulating PrP^{res} formation an unrelated peptide (canine parvo virus specific sequence peptide DGAVQPDGGQPAVRNER) was used in both testing setups described above. Addition of this peptide did not affect PrP^{res} formation in either of the two PMCA assay setups (data not shown) at various concentrations, indicating that the observed increases in PrP^{res} were a result of the specific PrP derived peptide amino acid sequences added to the reactions. Furthermore, PMCA assays were also performed for each peptide without scrapie positive homogenate, to determine whether *de novo* PrP^{res} could be formed when the PrP derived peptide was combined with PrP^{C}. Peptide was added at the optimum

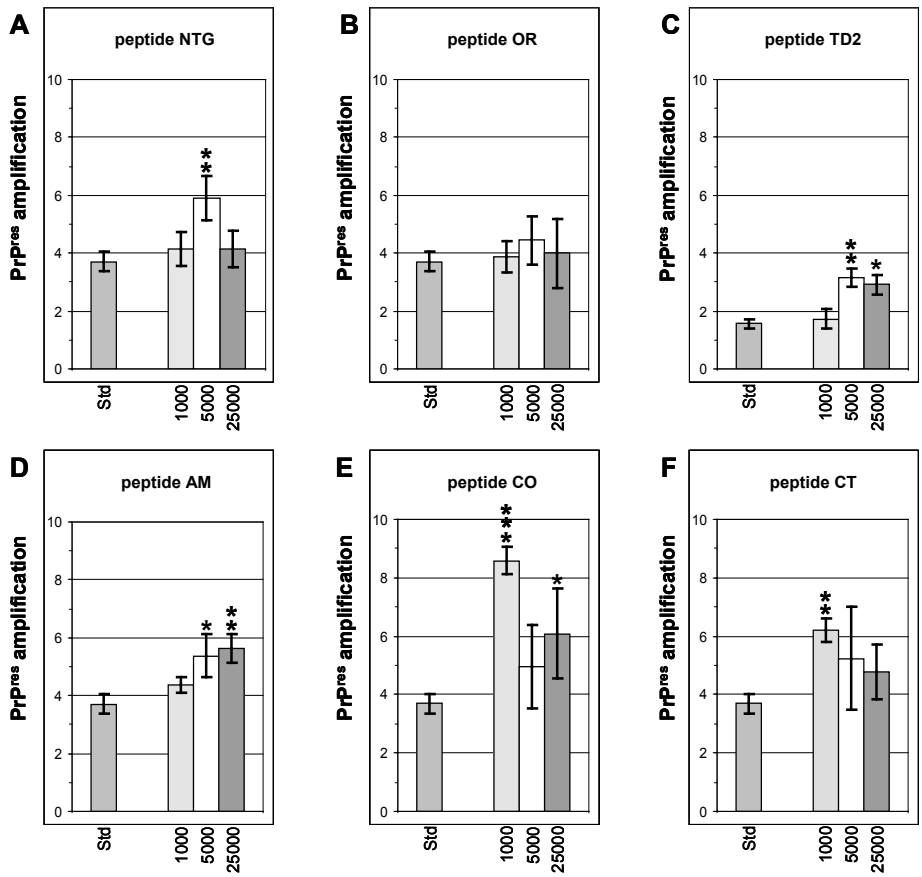


Figure 4. ***Effects of peptide pre-incubation on PrP^{res} formation in ovine PMCA.*** *Bar graphs depicting PrP^{res} formation of a standard PMCA reaction compared to a PMCA reaction in which the scrapie negative homogenate was pre-incubated with peptide with different molar excess amounts of peptide NTG (A), peptide OR (B), peptide TD2 (C), peptide AM (D), peptide CO (E) and peptide CT (F) before adding scrapie positive homogenate. For each peptide its specific corresponding standard is depicted (grey bar). The number of independent measurements (n), the median and s.e.m. for each peptide and molar excess are summarized in Table 2. An unpaired Student's t-test was performed to determine whether the PrP^{res} formation with peptide was significantly different to the corresponding standard PrP^{res} formation. P-values are listed in Table 2 and significant differences are marked; p-values between 0.05 and 0.001 with *, p-values between 0.001 and 0.0001 with ** and p-values ≤ 0.0001 with ***.*

(in the supplemented PMCA) molar excess for peptide NTG (1.000), OR (500) and AM (5.000). While peptides TD2, CO and CT were added at the highest molar excess (25.000) used in the in the PMCA assays described above. No significant conversion induced by either of these peptides was detected after PK digestion (data not shown), showing that only addition of scrapie-positive brain homogenate resulted in initiation of the conversion reaction.

Discussion.

In a previous study [15] we showed that ovine PrP binds to itself and mapped several domains using ovine (and bovine) prion protein derived peptide-arrays, yielding a PrP-specific binding pattern for soluble (monomeric) PrP that could be blocked by several PrP-specific monoclonal antibodies. The current study shows that the different PrP derived synthetic peptides exert different effects on binding in a peptide-array assay and on conversion of PrP^{C} to PrP^{res} in the PMCA assay. In order to better interpret the PMCA data, one needs to consider the effects of sonication in the PMCA. It may be expected that after the first incubation cycle, sonication simply results in shearing the elongated PrP^{Sc} which releases the peptide (Figure 5). This results in multiple seeds for the following incubation cycle so that after the first sonication cycle, conditions for both the pre-incubated and the supplemented PMCA can be considered identical. This is however not in agreement with the obtained results (discussed below). Therefore we have devised a more intricate schematic (Figure 6) to better account for the observed differences between the pre-incubated (Figure 6A) and supplemented PMCA (Figure 6B) for some of the peptides.

The conversion process is a succession of distinct steps, which most likely starts with the multimerisation of PrP^{C}. Wille *et al.* used electron crystallography to characterize the structure of two infectious variants of the prion protein [44]. By comparing projection maps of these two variants a model featuring β-helices was devised. This model was further refined by studying 119 all-β-folds observed in globular proteins [3]. It was proposed that PrP^{Sc} should adopt a β-sandwich, parallel β-helical architecture, or a parallel left-handed β-helical fold. This left-handed β-helical folded PrP can readily form trimers, providing a natural template for a trimeric model of PrP^{Sc} and another (similar) β-helical model was proposed, which largely explained species and strain-specificity [4]. In both models oligomerisation/trimerisation of PrP^{C} precedes initiation of conversion as depicted in Figure 7A, implying an important role for PrP^{C} self-interaction in the

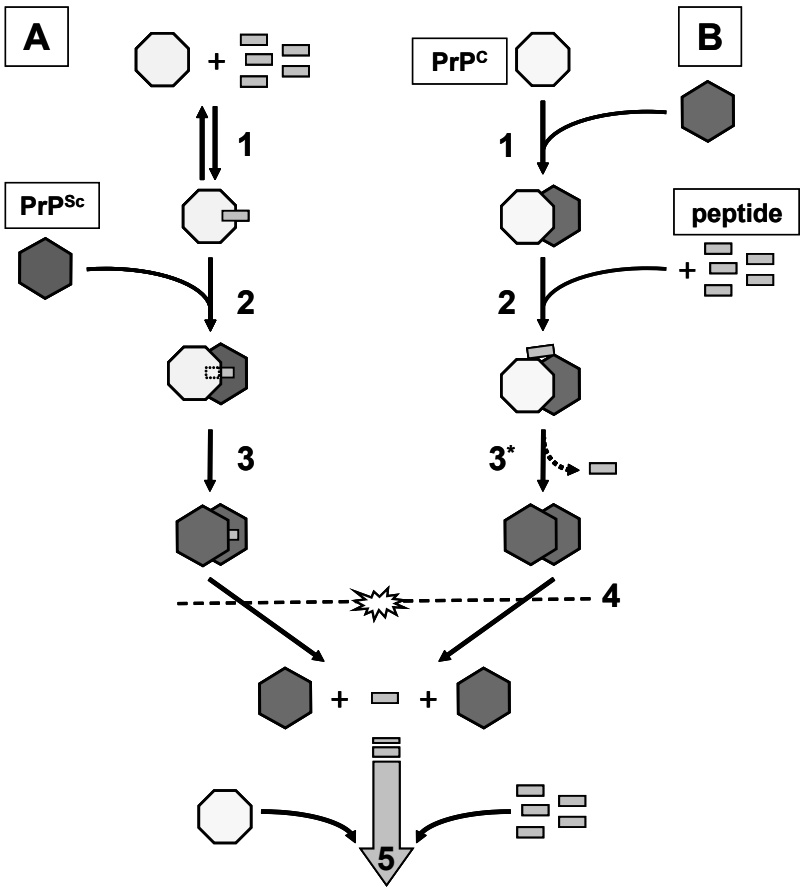
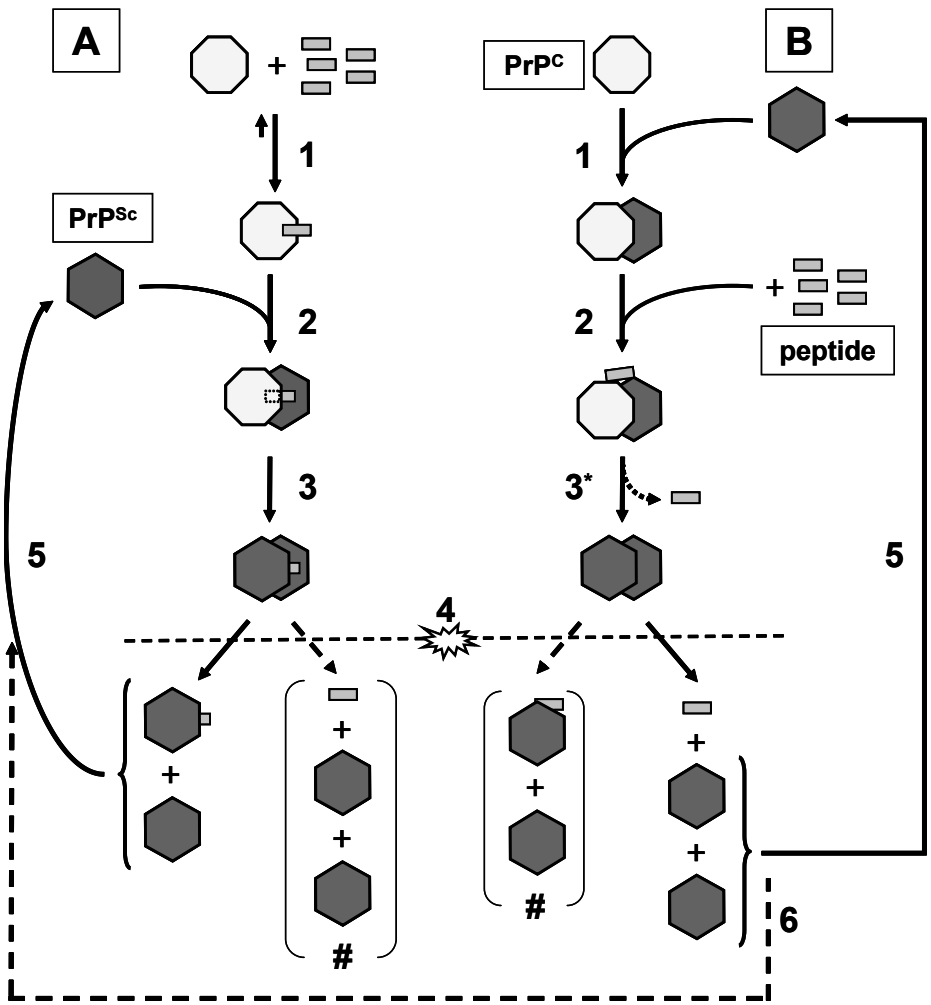


Figure 5. ***Schematic representation of one incubation/sonication cycle of PMCA.*** *Peptide is allowed to form a bond with PrP^C (1), resulting in all PrP^C binding peptide(s) (pre-incubation with an excess of peptide). The conversion reaction is initiated by adding PrP^{Sc} to the 'sensitized' PrP^C (2). It is possible that the peptide remains associated with the formed PrP^{res} during conversion (3). Sonication (4) shears PrP^{res}, but does not necessarily release peptide from 'sensitized' PrP^C and probably also not from the elongated PrP^{Sc} (#). The PrP^{res} fragments are in turn capable of recruiting and converting 'sensitized' PrP^C (5) during the next incubation cycle.*

Figure 6 (following page). ***Adapted schematic representation of one incubation/sonication cycle of PMCA. Pre-incubated PMCA (A)****: peptide is allowed to form a bond with PrP^C (1), resulting in all PrP^C binding peptide (pre-incubation with an excess of peptide). The conversion reaction is initiated by adding PrP^{Sc} to the 'sensitized' PrP^C (2). It is likely that the peptide is incorporated in formed PrP^{res} during conversion (3). Sonication (4) shears PrP^{res}, but does not release peptide from 'sensitized' PrP^C and probably not from the elongated PrP^{Sc} (#). The PrP^{res} fragments (some with peptide incorporated) are in turn capable of recruiting and converting 'sensitized' PrP^C (5) during the*

next incubation cycle. ***Supplemented PMCA (B)****: the conversion reaction is first initiated by combining* PrP^{C} *and* PrP^{Sc} *(1). Peptide is immediately added (2), which can bind to either* PrP^{C} *or* PrP^{Sc} *separately, but is probably only effective when binding both.* PrP^{C} *is converted, possibly releasing the peptide in the process (3). Sonication (4) shears* PrP^{res}*, probably releasing the peptide if it is still bound after step 3. The* PrP^{res} *fragments (unlikely with peptide incorporated, #) are in turn capable of recruiting and converting* PrP^{C} *(5) during the next incubation cycle. Even though* PrP^{C}*,* PrP^{res} *and peptide are all present after sonication, it is likely that fist the* PrP^{C}*-*PrP^{Sc} *complex is formed (1) before interaction with peptide (2) occurs. However, if the peptide is capable of 'sensitizing'* PrP^{C}*, the reaction proceeds as described for the pre-incubated PMCA reaction (6). After addition of all ingredients in the first cycle the pre-incubated -and supplemented PMCA reactions will have both unbound and bound peptide available for the following sonication-incubation cycles.*



conversion processes. Our data fits this multimerisation of PrP^{C}. Additionally, study of the amyloid-forming pathway revealed a pre-amyloid state containing partially unfolded monomers and dimers (PrP^{i}) [45-50]. Whether PrP^{i} is just the partially unfolded state (monomers and dimers) of PrP^{C} or whether it is a pre-formed trimer before further structural rearrangement towards PrP^{Sc} occurs (Figure 7B) remains to be elucidated. Conversion is initiated by recruitment of PrP to PrP^{Sc} (Figure 7C), after which PrP is (further) rearranged to adopt the tertiary structure of the PrP^{Sc} seed (Figure 7D). The elongated PrP^{Sc} is in turn capable to recruit and convert further PrP (Figure 7E). Taking these studies into account and their implications for conversion allows for a more detailed interpretation of the data presented in this study., Herein the peptide-array data is indicative for the effects on soluble PrP (PrP^{C}-PrP^{C} interaction), whereas the PMCA assay may be indicative for effects on PrP interactions (self- and PrP^{C}-PrP^{Sc} interaction) as well as interactions with chaperoning or inhibiting molecules. All six PrP-derived peptides tested affected conversion in either the supplemented and/or pre-incubated PMCA assay, whereas binding to the peptide-array was only completely abolished by three peptides [OR, TD2 and CT]. Taken together, this study shows that the previously determined self-interaction domains of PrP^{C} are of importance at several different phases in the conversion reaction.

Ovine peptide-array analysis previously revealed two high binding areas within PrP [15] of which the first high binding area encompasses the octarepeats, more specifically the consensus domain P(H)GG. This study showed that the octarepeat peptide (OR) was capable of blocking the binding pattern of PrP to the peptide-array, probably as a result of peptide-induced changes in the tertiary structure of the N-terminal tail and thus affecting PrP^{C} self-interaction. Only in the supplemented PMCA assay, a dose dependant and significant increase in PrP^{res} is observed. The octarepeats can modulate [51-55] but are not a necessity for the molecular processes underlying conversion [21, 56]. Interaction between PrP^{C} and PrP^{Sc} seems almost instantaneous [14], which would leave the peptide free to interact with the PrP^{C} - PrP^{Sc} complex as a whole or with co-factors present in the homogenates. Because the octarepeat stabilizes the interaction of PrP^{C} with the LRP-LR receptor [57], it seems that peptide OR indirectly affects the conversion process, either by affecting PrP^{i} stability/formation (Figure 7, B) or by stabilizing PrP^{i} interaction with PrP^{Sc} (Figure 7, C). Furthermore, a di-peptide containing the octarepeat self-aggregates into nanometric fibrils [58] and these may also be formed in the supplemented PMCA assay. Combined with our data, we propose that the flexible N-terminal tail containing the octarepeat region stabilizes PrP^{C}-PrP^{Sc} interaction during conversion and that free peptide OR forms nanometric fibrils mimicking and increasing PrP^{C}-PrP^{Sc} stabilization, thus aiding subsequent conversion. Also part of the peptide-array first high binding area

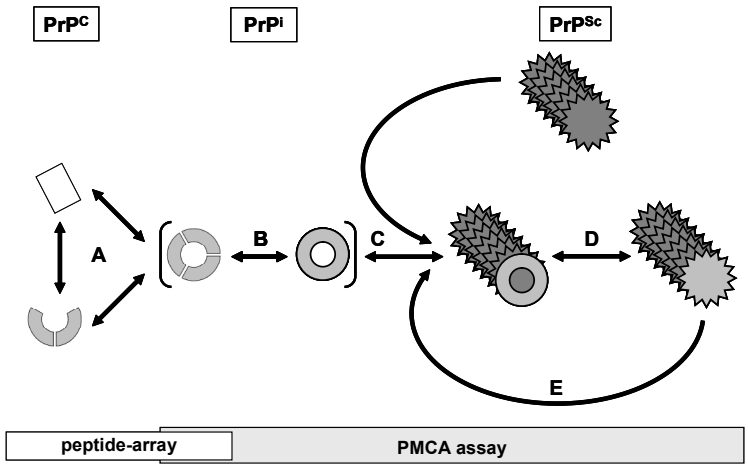


Figure 7. ***Schematic representation of likely steps in prion protein conversion.*** *Preceding conversion a multimer of PrP^C molecules is formed [A]. Possibly a dimer is formed and both monomeric -and dimeric PrP is partially refolded before forming the structural subunit of a fibril; trimeric PrP. PrP^C may however first form a trimer before partially unfolding and/or refolding. Partial unfolding/refolding is at the basis of forming an intermediate isoform of PrP (PrP^i) [B]. PrP^i can subsequently interact with the conversion seed (PrP^{Sc}) [C], which allows conversion of PrP into PrP^{Sc}. Several PrP^i may have to be 'stacked' onto the conversion seed before conversion of the first PrP^i bound can occur. During conversion PrP^i is further refolded into PrP^{Sc}, its tertiary structure adapted to that of the conversion seed (D). The newly elongated PrP^{Sc} in turn can act as a seed for further conversion of PrP (E). The bars beneath the schematic denote for which conversion processes the peptide-array results (white bar) and PMCA assay results (shaded bar) are indicative.*

is binding domain 2 ([102-WNK-104], Figure 1) and the data presented here shows that pre-incubation of the peptide TD2 (containing [102-WNK-104]) with PrP abolished binding of PrP to the peptide-array. This indicates the importance of this domain in PrP^C self-interaction (Figure 7A and possibly 7B). Increased PrP^{res} production was observed in both the supplemented –and the pre-incubated PMCA-assay. The mechanism by which the peptide stimulates PrP^{res} formation may simply be due to peptide enhanced interaction between separate PrP molecules. Alternatively, peptide TD2 could aid unfolding and/or refolding of PrP^C during conversion; binding domain 2 [102-WNK-104], together with the amyloidogenic motif, is part of the region of PrP^C that is partially unfolded and refolded during oligomerization of PrP^C into a β-sheet-rich soluble isoform of PrP [47].

The second high binding area in the peptide-array contains the domain [140-PLIHFGNDY-148] (domain 3, Figure 1). This study shows that the peptide CO containing the domain [140-PLIHFGNDY-148] does not affect binding of PrP to the peptide-array at all, thereby ruling out direct involvement in PrP^{C} self-interaction. Several studies have established that polymorphisms at sheep PrP amino acid position 136, 154 and 171 surrounding [140-PLIHFGNDY-148] are most relevant in differential TSE susceptibility [5, 59-63] and that stability of this PrP region is a crucial determinant in whether PrP^{C} is converted [40, 64] (affecting species-barrier and/or scrapie susceptibility). Therefore peptide CO induced PrP^{res} formation in the pre-incubated PMCA assay is either due to this core region peptide facilitating binding of PrP^{C} to PrP^{Sc} or the peptide affects the stability of this region of PrP^{C} (interacting with the 'self-domain' or another domain of PrP) thereby facilitating refolding of PrP.

The N-terminal peptide NTG (containing the glycosaminoglycan binding motif) only moderately affects binding of PrP^{C} throughout the peptide-array, suggesting that interaction of the peptide with PrP^{C} results either in slight changes in the tertiary structuring affecting solubility of PrP^{C} or in diminished availability of the previously determined domains [15] for interaction with the peptide-array. Intriguingly, peptide NTG induces PrP^{res} formation in both the supplemented (dose dependant) and pre-incubated (dose optimum) PMCA assay. The glycosaminoglycan heparan sulphate proteoglycan (HSPG) and pentosan polysulphate (PPS) stimulate PrP^{res} formation *in vitro* and suggests that free glycosaminoglycans acted as a contact-mediator allowing interaction of PrP^{C} and PrP^{Sc} [65]. The N-terminal peptide NTG likely indirectly affects *in vitro* conversion either by mimicking glycosaminoglycan binding to domain [27-RPKPGGG-33] or by recruiting glycosaminoglycans onto PrP^{C}, facilitating conversion of PrP^{C} into new PrP^{Sc} after seeding. These studies and our PMCA assay data strongly implicate glycosaminoglycans as an important cofactor in the conversion process.

The ability of peptide AM, which encompasses the amyloidogenic motif [116-AGAAAAGA-123], to moderately block binding of PrP to the peptide-array was somewhat surprising, since we previously showed that the amyloidogenic motif was not involved in PrP self-interaction [15]. Peptide AM mainly inhibits binding of PrP to the peptides covering the N-terminal part of the mature PrP protein, suggesting that peptide AM interacts with one (or more) of the other previously determined binding domains. In contrast to earlier reports [41, 66, 67], we observed that peptide AM (containing the amyloidogenic motif) slightly but significantly increased PrP^{res} formation in both the pre-incubated –and supplemented PMCA. However, all these inhibiting peptides contained two or more additional amino acids of the putative aggregation sites (flanking

the amyloidogenic motif) implicated in aggregation/oligomerisation [68], suggesting inhibition by these peptides is due to interference with aggregation/oligomerization. Additionally, differences between the used conversion systems (i.e. availability of cofactors) are likely to play a role as well. The peptide AM used in this study specifically focuses only on the amyloidogenic motif. Our data suggests that peptide AM interacts with the N-terminal tail of PrP^{C} (octarepeat motif or [102-WNK-104]), probably altering its tertiary structure and facilitating the proposed stabilizing effect of the N-terminal tail. Alternatively, peptides containing only the amyloidogenic motif are also capable of forming a β-sheet rich layer at the water-air interface when sonicated [69] and peptide AM may form a β-sheeted backbone that interacts with the PrP^{C}-PrP^{Sc} complex, mimicking and/or complementing the proposed stabilizing effect of the N-terminal tail.

Peptide CT overlaps most of the third alpha helix of PrP^{C} as well as the second glycosylation site and the second cysteine involved in the di-sulphide bridge formed in PrP^{C}. The capacity to completely block PrP binding to the peptide-array suggests that the domain [225-SQAY-228] is of importance in PrP^{C} self-interaction. This study shows a slight significant increase in PrP^{res} formation when peptide CT is pre-incubated with scrapie negative brain homogenate. This contradicts results using a similar peptide capable of inhibiting cell free conversion [41]. However, this inhibiting peptide is four amino acids larger than peptide CT, which may account for the difference in effects and/or it may just be due to the differences in experimental technique between the cell free conversion and the PMCA assay. This seems to be corroborated by the observation that in the supplemented PMCA (setup closest resembling conditions in cell free conversion [41]) peptide CT seems to slightly inhibit PrP^{res} formation albeit not significantly. Fibrillization of a human PrP peptide fragment is hindered by disulfide bridge formation between two peptides [70] or when an additional disulfide bridge is introduced [34], which indicates that peptide CT (when pre-incubated with PrP^{C}) likely compromises the disulfide bridge, destabilizing PrP^{C}, which consequently promotes trimerization or formation of a conversion intermediate (Figure 7B) and thus facilitating conversion.

In the PMCA-assay all peptides revealed an inducing effect on PrP^{res} formation in the supplemented –and/or pre-incubated PMCA-assay. Above possible explanations for these effects have been discussed for each peptide. However it can not be ruled out that the peptides may have had an opposite effect; instead of interacting with PrP, peptide may have interacted with possible conversion inhibitory factors present in the homogenate, thus indirectly allowing conversion to take place more efficiently. Identifying these possible 'natural' inhibitory factors may prove an alternative line of investigation towards the underlying mechanisms involved in prion replication and may provide additional targets for future prion therapy.

Conclusions.

The binding domains found for ovine PrP^C using a prion protein peptide-array are primarily indicative of prion protein self-interaction. Apparently several specific self-interactions between individual PrP molecules occur, which include both PrP^C-PrP^C as well as PrP^C-PrP^{Sc} interactions. The data presented here imply an influence of binding domain [140-PLIHFGNDY-148] on the stability of the region of PrP previously determined to be involved in the species-barrier and/or susceptibility to scrapie. Furthermore our data indicates a stabilizing function for the octarepeats region (N-terminal tail) in PrP^C-PrP^{Sc} interaction and thus improving subsequent conversion. Our data further suggests that the N-terminal glycosaminoglycan binding motif [27-RPKPGGG-33] affects the conversion process indirectly, and implicates glycosaminoglycans as an important cofactor in prion disease pathogenicity. Peptide AM containing the amyloidogenic motif indirectly affects conversion either by aiding and/or complementing the proposed stabilizing function of the N-terminal tail of PrP^C. Finally, the data implicates direct involvement of the two binding domains [102-WNK-104] and [225-SQAY-228] in self-interaction between PrP^C molecules preceding binding to PrP^{Sc} and subsequent conversion. Therefore these two domains may prove prime targets for development of new therapeutic strategies. Our results emphasize the importance of the stability of the PrP^C-PrP^C and PrP^C-PrP^{Sc} interactions in PrP conversion, which is an essential determinant in the effects of disease associated mutations, as well as the species-barrier. Focussing on the (stabilizing) self-interaction domains of PrP and the subsequent conversion processes may lead to further therapeutic strategies with the possibility to leave the physiological function of the prion protein unaffected.

Methods.

MBP-PrP construction, expression and purification.

The mature part of sheep PrP (ARQ) open reading frame (ORF) was cloned into the pMAL Protein Fusion and Purification System (New England Biolabs) as described before [15], resulting in the maltose binding protein (MBP) fusion to the N-terminus of PrP (MBP-PrP). MBP-PrP was expressed and purified by affinity chromatography as described in the manual of the pMAL Protein Fusion and Purifications System (method I; New England Biolabs) To improve binding of MBP-PrP and to prevent formation of interchain disulfide upon lysis (as suggested in the protocol), β-mercaptoethanol was added. Quantity and quality of the eluted MBP-PrP was determined before use in the peptide-array by SDS-PAGE (12% NuPAGE, Invitrogen). After separation the gel was

either stained with Sypro Orange (total protein stain, Molecular Probes) or analyzed by Western blotting and immunodetection of MBP-PrP with polyclonal antiserum R521-7 specific for PrP [71].

Peptides.

Peptides were synthesized with an acetylated N-terminus and an amidated C-terminus as described before [71]. The synthesized peptides were purified by high performance liquid chromatography using mass spectrometric analysis for identification. The resulting purified peptides were at least 90% pure. All peptides dissolved well in water and solutions were stored frozen. Sequential properties like iso-electric point were calculated with the Peptide Property Calculator made available online in the tools section of Innovagen (www.innovagen.se).

Peptide-array analysis.

Synthesis of complete sets of overlapping 15-mer peptides were carried out on grafted plastic surfaces, covering the ovine PrP amino acid sequence of mature PrP (residues 25-234 [72]). Coupling of the peptides to the plastic surface consisting of a 455-well credit-card size plastic (minicard) and subsequent ELISA analyses including subsequent background correction, relative density value calculation and binding pattern interpretation were performed as described before [15, 73]. This study also showed that linking of MBP to PrP did not have any disadvantageous effects and therefore its properties are indicative for PrP. Peptide blocking studies were performed by pre-incubating the MBP-PrP with molar excesses of prion peptides before incubating the PrP-peptide mixture as the antigen on the minicard. Binding to the peptide-array was considered relevant when at least at 3 consecutive peptides optical density values of at least 2.5 times the background were observed.

Protein Misfolding Cyclic Amplification assay.

The protein misfolding cyclic amplification (PMCA) assay first described by Saborio *et al.* [9] and has been shown applicable to amplify PrP^{Sc} from different sources [10, 43]. In short; a 10% brain homogenate from a (confirmed) scrapie-positive sheep (SPH) was diluted with 50-100 times in 10% (confirmed) scrapie-negative sheep brain homogenate (SNH) after which the reaction was subjected to one round of sonication-incubation cycles (24 hours; 48 cycles). To test the influence of the peptides on conversion, the peptides were either added after combining the scrapie positive –and negative brain homogenates (supplemented PMCA) or peptide was pre-incubated with the scrapie-negative homogenate (pre-incubated PMCA) before addition of the scrapie-positive brain homogenate. The total amount of PrP^{C} in the PMCA reaction was calculated based on the quantification of the amount of PrP^{C} in brain tissue [74]. The amount of peptide needed for a reaction was calculated based on the total amount of PrP^{C} in the reaction, adjusted for the size

difference between peptide and PrP^C, so that the amount of peptide added represents various molar excesses of peptide molecules relative to the total number of PrP^C molecules present in the reaction. The amount of formed PrP^{res} was determined after proteinase K digestion (100 μg/ml) of the PMCA reaction and analysis by SDS-PAGE and subsequent Western blotting. Standard detection of PrP^{res} was performed with monoclonal antibody 9A2 [75], except for PMCA reactions containing peptide SAU14, which contains the binding epitope of 9A2. In this case we used a proven combination of monoclonal antibodies L42 [76] and Sha31 [77]. To determine whether the determined amounts of PrP^{res} were significantly different in comparison to its corresponding standard PMCA-assay reaction, an unpaired Student's t-test was performed and the p-value calculated.

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References.

1. DeArmond, S.J. and S.B. Prusiner, *Perspectives on prion biology, prion disease pathogenesis, and pharmacologic approaches to treatment.* Clin Lab Med, 2003. 23(1): p. 1-41.
2. Bossers, A., et al., *In vitro conversion of normal prion protein into pathologic isoforms.* Clin Lab Med, 2003. 23(1): p. 227-47.
3. Govaerts, C., et al., *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A, 2004. 101(22): p. 8342-7.
4. Langedijk, J.P., et al., *Two-rung Model of a Left-handed beta-Helix for Prions Explains Species Barrier and Strain Variation in Transmissible Spongiform Encephalopathies.* J Mol Biol, 2006. 360(4): p. 907-920.
5. Bossers, A., R. de Vries, and M.A. Smits, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol, 2000. 74(3): p. 1407-14.
6. Kocisko, D.A., et al., *Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier.* Proc Natl Acad Sci U S A, 1995. 92(9): p. 3923-7.
7. Castilla, J., et al., *Protein misfolding cyclic amplification for diagnosis and prion propagation studies.* Methods Enzymol, 2006. 412: p. 3-21.
8. Castilla, J., P. Saa, and C. Soto, *Detection of prions in blood.* Nat Med, 2005. 11(9): p. 982-5.
9. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. 411(6839): p. 810-3.
10. Soto, C., et al., *Pre-symptomatic detection of prions by cyclic amplification of protein misfolding.* FEBS Lett, 2005. 579(3): p. 638-42.
11. Dubois, M.A., et al., *Multiplicative genetic effects in scrapie disease susceptibility.* C R Biol, 2002. 325(5): p. 565-70.
12. Tranulis, M.A., *Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep.* Apmis, 2002. 110(1): p. 33-43.
13. Sabuncu, E., et al., *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol, 2003. 77(4): p. 2696-700.
14. Rigter, A. and A. Bossers, *Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion.* J Gen Virol, 2005. 86(Pt 9): p. 2627-34.
15. Rigter, A., et al., *Mapping of possible prion protein self interaction domains using peptide arrays.* BMC Biochem, 2007. 8(1): p. 6.
16. Pan, T., et al., *Cell-surface prion protein interacts with glycosaminoglycans.* Biochem J, 2002. 368(Pt 1): p. 81-90.

17. Brown, D.R., *Prion protein expression aids cellular uptake and veratridine-induced release of copper.* J Neurosci Res, 1999. 58(5): p. 717-25.
18. Brown, D.R., *Role of the prion protein in copper turnover in astrocytes.* Neurobiol Dis, 2004. 15(3): p. 534-43.
19. Brown, D.R., C. Clive, and S.J. Haswell, *Antioxidant activity related to copper binding of native prion protein.* J Neurochem, 2001. 76(1): p. 69-76.
20. Dupuis, L., et al., *Loss of prion protein in a transgenic model of amyotrophic lateral sclerosis.* Mol Cell Neurosci, 2002. 19(2): p. 216-24.
21. Flechsig, E., et al., *Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice.* Neuron, 2000. 27(2): p. 399-408.
22. Frankenfield, K.N., E.T. Powers, and J.W. Kelly, *Influence of the N-terminal domain on the aggregation properties of the prion protein.* Protein Sci, 2005. 14(8): p. 2154-66.
23. Klamt, F., et al., *Imbalance of antioxidant defense in mice lacking cellular prion protein.* Free Radic Biol Med, 2001. 30(10): p. 1137-44.
24. Miele, G., et al., *Ablation of cellular prion protein expression affects mitochondrial numbers and morphology.* Biochem Biophys Res Commun, 2002. 291(2): p. 372-7.
25. Pauly, P.C. and D.A. Harris, *Copper stimulates endocytosis of the prion protein.* J Biol Chem, 1998. 273(50): p. 33107-10.
26. Rachidi, W., et al., *Expression of prion protein increases cellular copper binding and antioxidant enzyme activities but not copper delivery.* J Biol Chem, 2003. 278(11): p. 9064-72.
27. Sakudo, A., et al., *Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line.* Biochem Biophys Res Commun, 2003. 308(3): p. 660-7.
28. Walz, R., et al., *Increased sensitivity to seizures in mice lacking cellular prion protein.* Epilepsia, 1999. 40(12): p. 1679-82.
29. Wong, B.S., et al., *Differential contribution of superoxide dismutase activity by prion protein in vivo.* Biochem Biophys Res Commun, 2000. 273(1): p. 136-9.
30. Wong, E., A.M. Thackray, and R. Bujdoso, *Copper induces increased beta-sheet content in the scrapie-susceptible ovine prion protein PrPVRQ compared with the resistant allelic variant PrPARR.* Biochem J, 2004. 380(Pt 1): p. 273-82.
31. Zeng, F., et al., *Tethering the N-terminus of the prion protein compromises the cellular response to oxidative stress.* J Neurochem, 2003. 84(3): p. 480-90.
32. Jacobs, J.G., et al., *Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe.* J Clin Microbiol, 2007. 45(6): p. 1821-9.

33. Thuring, C.M., et al., *Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein.* J Clin Microbiol, 2004. 42(3): p. 972-80.
34. Knowles, T.P. and R. Zahn, *Enhanced stability of human prion proteins with two disulfide bridges.* Biophys J, 2006. 91(4): p. 1494-500.
35. Korth, C., et al., *Prion (PrPSc)-specific epitope defined by a monoclonal antibody.* Nature, 1997. 390(6655): p. 74-7.
36. Kuczius, T., et al., *Binding of N- and C-terminal anti-prion protein antibodies generates distinct phenotypes of cellular prion proteins (PrPC) obtained from human, sheep, cattle and mouse.* Febs J, 2007. 274(6): p. 1492-502.
37. Martucci, F., et al., *Detection of typical and atypical BSE and scrapie prion strains by prion protein motif-grafted antibodies.* J Gen Virol, 2009.
38. Moroncini, G., et al., *Motif-grafted antibodies containing the replicative interface of cellular PrP are specific for PrPSc.* Proc Natl Acad Sci U S A, 2004. 101(28): p. 10404-9.
39. Moroncini, G., et al., *Pathologic prion protein is specifically recognized in situ by a novel PrP conformational antibody.* Neurobiol Dis, 2006. 23(3): p. 717-24.
40. Paramithiotis, E., et al., *A prion protein epitope selective for the pathologically misfolded conformation.* Nat Med, 2003. 9(7): p. 893-9.
41. Horiuchi, M., et al., *Inhibition of interactions and interconversions of prion protein isoforms by peptide fragments from the C-terminal folded domain.* J Biol Chem, 2001. 276(18): p. 15489-97.
42. Solforosi, L., et al., *Toward Molecular Dissection of PrPC-PrPSc Interactions.* J Biol Chem, 2007. 282(10): p. 7465-71.
43. Soto, C., G.P. Saborio, and L. Anderes, *Cyclic amplification of protein misfolding: application to prion-related disorders and beyond.* Trends Neurosci, 2002. 25(8): p. 390-4.
44. Wille, H., et al., *Structural studies of the scrapie prion protein by electron crystallography.* Proc Natl Acad Sci U S A, 2002. 99(6): p. 3563-8.
45. Apetri, A.C., et al., *Early intermediate in human prion protein folding as evidenced by ultrarapid mixing experiments.* J Am Chem Soc, 2006. 128(35): p. 11673-8.
46. Apetri, A.C., K. Surewicz, and W.K. Surewicz, *The effect of disease-associated mutations on the folding pathway of human prion protein.* J Biol Chem, 2004. 279(17): p. 18008-14.
47. Gerber, R., et al., *Oligomerization of the human prion protein proceeds via a molten globule intermediate.* J Biol Chem, 2007. 282(9): p. 6300-7.
48. Hosszu, L.L., et al., *Conformational properties of beta -PrP.* J Biol Chem, 2009.
49. Jenkins, D.C., I.D. Sylvester, and T.J. Pinheiro, *The elusive intermediate on the folding pathway of the prion protein.* Febs J, 2008. 275(6): p. 1323-35.

50. Stohr, J., et al., *Mechanisms of prion protein assembly into amyloid.* Proc Natl Acad Sci U S A, 2008. 105(7): p. 2409-14.
51. Campbell, T.A., et al., *A prion disease with a novel 96-base pair insertional mutation in the prion protein gene.* Neurology, 1996. 46(3): p. 761-6.
52. Cochran, E.J., et al., *Familial Creutzfeldt-Jakob disease with a five-repeat octapeptide insert mutation.* Neurology, 1996. 47(3): p. 727-33.
53. Goldfarb, L.G., et al., *A new (two-repeat) octapeptide coding insert mutation in Creutzfeldt-Jakob disease.* Neurology, 1993. 43(11): p. 2392-4.
54. Goldfarb, L.G., et al., *Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene.* Proc Natl Acad Sci U S A, 1991. 88(23): p. 10926-30.
55. Laplanche, J.L., et al., *Two novel insertions in the prion protein gene in patients with late-onset dementia.* Hum Mol Genet, 1995. 4(6): p. 1109-11.
56. Lawson, V.A., et al., *Flexible N-terminal region of prion protein influences conformation of protease-resistant prion protein isoforms associated with cross-species scrapie infection in vivo and in vitro.* J Biol Chem, 2004. 279(14): p. 13689-95.
57. Hundt, C., et al., *Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor.* Embo J, 2001. 20(21): p. 5876-86.
58. Madhavaiah, C. and S. Verma, *Self-aggregation of reverse bis peptide conjugate derived from the unstructured region of the prion protein.* Chem Commun (Camb), 2004(6): p. 638-9.
59. Bossers, A., *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie.* 1999, University of Utrecht.
60. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.
61. Bossers, A., et al., *PrP genotype contributes to determining survival times of sheep with natural scrapie.* J Gen Virol, 1996. 77 (Pt 10): p. 2669-73.
62. Goldmann, W., et al., *PrP genotypes and the Sip gene in Cheviot sheep form the basis for scrapie strain typing in sheep.* Ann N Y Acad Sci, 1994. 724: p. 296-9.
63. Hunter, N., et al., *Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes.* Arch Virol, 1996. 141(5): p. 809-24.
64. Surewicz, W.K., E.M. Jones, and A.C. Apetri, *The emerging principles of Mammalian prion propagation and transmissibility barriers: insight from studies in vitro.* Acc Chem Res, 2006. 39(9): p. 654-62.
65. Wong, C., et al., *Sulfated glycans and elevated temperature stimulate PrP(Sc)-dependent cell-free formation of protease-resistant prion protein.* Embo J, 2001. 20(3): p. 377-86.

66. Chabry, J., B. Caughey, and B. Chesebro, *Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides.* J Biol Chem, 1998. 273(21): p. 13203-7.

67. Chabry, J., et al., *Species-independent inhibition of abnormal prion protein (PrP) formation by a peptide containing a conserved PrP sequence.* J Virol, 1999. 73(8): p. 6245-50.

68. Ziegler, J., et al., *Putative aggregation initiation sites in prion protein.* FEBS Lett, 2006. 580(8): p. 2033-40.

69. Satheeshkumar, K.S. and R. Jayakumar, *Sonication induced sheet formation at the air-water interface.* Chem Commun (Camb), 2002(19): p. 2244-5.

70. Bosques, C.J. and B. Imperiali, *The interplay of glycosylation and disulfide formation influences fibrillization in a prion protein fragment.* Proc Natl Acad Sci U S A, 2003. 100(13): p. 7593-8.

71. van Keulen, L.J., et al., *Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie.* Vet Pathol, 1995. 32(3): p. 299-308.

72. Goldmann, W., et al., *Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon.* J Gen Virol, 1991. 72 (Pt 1): p. 201-4.

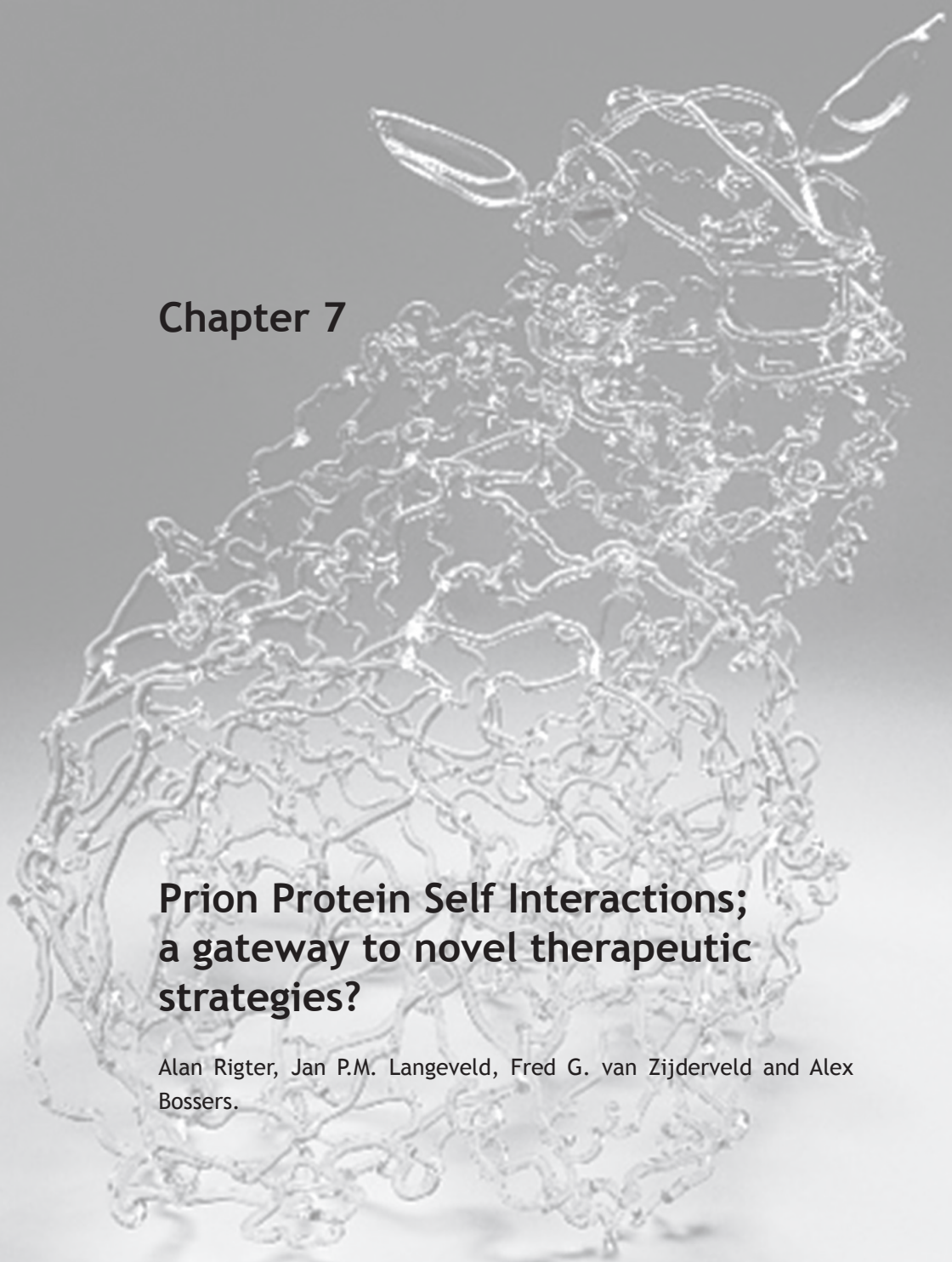
73. Rigter, A., et al., *Mapping functional prion-prion protein interaction sites using prion protein based peptide-arrays.* Methods Mol Biol, 2009. 570: p. 257-71.

74. Moudjou, M., et al., *Cellular prion protein status in sheep: tissue-specific biochemical signatures.* J Gen Virol, 2001. 82(Pt 8): p. 2017-24.

75. Langeveld, J.P., et al., *Rapid and discriminatory diagnosis of scrapie and BSE in retropharyngeal lymph nodes of sheep.* BMC Vet Res, 2006. 2: p. 19.

76. Harmeyer, S., E. Pfaff, and M.H. Groschup, *Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants.* J Gen Virol, 1998. 79 (Pt 4): p. 937-45.

77. Feraudet, C., et al., *Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells.* J Biol Chem, 2005. 280(12): p. 11247-58.



Chapter 7

Prion Protein Self Interactions; a gateway to novel therapeutic strategies?

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Prion Protein Self Interactions; a gateway to novel therapeutic strategies?

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Abstract.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurodegenerative disorders and include among others Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep. The central event in disease development in TSEs is the refolding of the normal host-encoded cellular prion protein (PrP) into abnormal and disease associated prion protein. The agent is thought to consist mainly or exclusively of these pathologically folded PrP molecules. The exact molecular mechanisms underlying this process and the role of normal PrP in the conversion to pathological isoforms of PrP are still poorly understood. The highly conserved PrP gene structure and organisation however, suggests that its function is important, even though PrP knockout mice appear to develop normally.

Conversion of normal PrP is initiated by interaction with abnormal PrP (or "agent") resulting in refolding of normal PrP into new pathological PrP ("agent replication"). Normal PrP was shown to interact/bind with many different molecules including metal ions, nucleic acids, several (receptor) proteins, and the prion protein itself. The processes underlying agent replication (normal to abnormal PrP conversion) are most likely initiated by selective interaction between PrP molecules and potentially influenced by chaperone molecules.

Thus far no vaccine, disease reversing therapeutic compounds or strategies (cure) exists, although there are some compounds capable of slowing the progression of prion disease. Studies towards interference to date have primarily focussed on inference with the interaction between normal and pathological isoforms of PrP in order to develop therapeutic strategies or find compounds capable of inhibiting prion propagation. Most described strategies are either directed at depletion of normal PrP and thus preventing pathological PrP formation and accumulation, or are based on preventing interaction between normal and abnormal PrP. Other therapeutic strategies focus on selective (self) interaction of normal PrP molecules. Increased understanding of these interactions and the processes in which normal PrP plays a (active) role, could potentially lead to new modes of inhibiting prion protein conversion in which the physiological function(s) of normal PrP is retained. Ultimately this may lead to therapeutic strategies that are effective not only as a prophylactic but also in later stages of prion disease development. Here we review the data underlying these PrP-based approaches.

Introduction.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurodegenerative disorders affecting both humans and animals. TSEs or prion diseases can occur as an infectious, sporadic or hereditary disease and includes (among others) Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. Even though the mechanisms by which these disorders cause disease are not well defined, the common factor between these disorders is the accumulation of the disease associated prion protein (PrP^{Sc}) mainly in neuronal tissues. During disease development the host-encoded cellular prion protein (PrP^{C}) is refolded (also known as conversion) into the disease associated isoform PrP^{Sc}, which possesses biochemical properties distinct from PrP^{C}. This change in biochemical properties leads to deposition of PrP^{Sc} and consequently causes cell-death resulting in sponge-like degeneration of neuronal tissue (hence the term spongiform). The onset of symptoms usually occurs a long time after infection (several years), therefore diagnosis typically is only possible in the last stages of prion disease development. Symptoms of TSEs vary, but include behavioural changes, (gradual) loss of motor skills and mental impairment. To date definitive diagnosis can only be achieved by post-mortem microscopic analysis of the brain.

The first to describe the possibility of an infectious protein as the causative agent in scrapie was Griffith [1], but the term prion (a derivative of proteinacious infectious particle) and its formal definition was introduced by Prusiner [2]. Since the 1988 outbreak of BSE in the United Kingdom, research into prion disorders has increased significantly, especially since the emergence of a new variant of Creutzfeldt-Jakob disease in 1996 (vCJD [3]) which is believed to be the result of the consumption of BSE infected beef or other catlle products. Even though the feared epidemic of vCJD has not taken on biblical proportions (thus far), the long term effects of the BSE outbreak is difficult to predict, especially when taken in account that TSE disorders usually have specific genetic aetiology and are characterized by a long incubation period (several years, even decades) before disease onset.

The exact physiological function of PrP^{C} and molecular mechanisms underlying PrP conversion remain elusive. However modelling data based upon research observations reveal an important role for PrP self-interaction (di –and trimerization of PrP^{C} before conversion by PrP^{Sc}). This merits studies focussing on interactions between PrP molecules as well as between PrP and other proteins or molecules to ultimately find effective modes for intervening therapeutic strategies. To date a few different therapeutic

compounds have been studied, which unfortunately have not resulted in a cure of TSE or even a substantial health improvement. This review will discuss some of the interaction partners of the prion protein, distinguishing between partners likely involved in 'regular' prion protein biology and interaction partners that help elucidate the physiological function of PrP^{C}, as well as homologues that may provide additional clues about the physiological function. In light of our work on determining self-interaction domains of PrP and their effects on the prion conversion processes, the pros and cons of the most promising therapeutic compounds are discussed, as well as some previously unpublished work on alternatives to therapies currently in development are presented.

The prion protein.

Cellular prion protein.

Prion protein is a cellular C-terminal glycosylphosphatidyl inositol (GPI) anchored protein which is highly conserved among mammalian species. In its mature form it is anchored in the plasma membrane, with the majority of its N-terminal chain pointing into the extracellular space. The biosynthetic pathway of PrP^{C} can be compared to that of other membrane- and secreted proteins [4,5]. The GPI-anchor signal sequence is also thought to have the additional function of aiding in translocation of PrP^{C} to caveolae-like domains on the surface of the cell [6]. Next to the 'normally' secreted and GPI-anchored PrP^{C} potential transmembrane variants can be expressed, which make up <10% of the total PrP^{C} expressed. Inherited and infectious prion diseases have been shown to have a common pathway for neurodegeneration [7] and several lines of investigation have led to the hypothesis that increased expression of the C-terminal transmembrane variant (^{Ctm}PrP) is responsible for the neurotoxic effects seen in prion diseases, either due to disease related mutations or by PrP^{Sc} induced ^{Ctm}PrP expression [8], explaining why neurodegenerative effects may be seen without accumulation of PrP.

Disease associated prion protein.

The central event in prion disease is the partial unfolding of PrP^{C} and refolding into the disease associated PrP^{Sc}. Even though PrP^{C} and PrP^{Sc} have the same origin, distinct biochemical differences are apparent between these two forms of the same protein. PrP^{C} is by definition soluble and can be fully degraded by proteinase K (PK). On the other hand, PrP^{Sc} is insoluble even in mild detergents and forms precipitates or aggregates (including amyloid plaques or fibrils, reviewed in [9]). In general differences occur within the

secondary, tertiary and quaternary structure. PrP^{C} usually is a monomeric protein that consists of mainly α-helical structure (42%) and is considered the non-infectious form of PrP, whereas PrP^{Sc} is a polymeric aggregate that has a lower α-helix (20%) and higher β-sheet (34%) content compared to PrP^{C} and is considered to be the infectious form of PrP.

Route of infection.

Infectious prion diseases like scrapie in sheep, BSE in cattle and vCJD in humans typically occur by the oral route. This route necessitates primary replication of the infectious agent in a peripheral compartment prior to brain invasion (reviewed in [10,11]). After oral infection PrP^{Sc} can be found in Peyer's patches in the gut and is followed by prion propagation to splenic lymphoid tissue and/or in gut-associated lymphoid tissue (GALT; including tonsil), resulting in prion transport by splenic innervation to the brainstem and spinal cord. In sheep the route of transfection is well defined and progression (in time) is defined by three phases; the first phase is after ingestion is invasion of the gut associated lymphoid tissues (GALT). The next phase is infection of non-GALT lymphoid tissues or lymphatic dissimination has taken place. The final phase is neuroinvasion likely through infection of the enteric nervous system, followed by infection of the parasympathetic and sympathetic efferent neuronal pathways resulting in infection of the spinal cord and brain. The route of infection seems similar for scrapie in sheep and BSE in both cattle and sheep [12,13]. However there are some differences; whereas cattle seem to be the end-host for BSE, scrapie in sheep is endemic.

Physiological function of PrP^{C}.

In order to devise effective therapeutic strategies, knowledge on prion protein biology and physiological function is imperative. Understanding the physiological function(s) of PrP^{C} may lead to determining pathways where PrP^{C} fulfils a pivotal role, which in turn may provide alternative targets for therapeutic approaches. Expression of PrP^{C} is most abundant in the brain, but has also been detected in other non-neuronal tissues including lymphoid cells and the gastrointestinal tract [14]. The exact physiological function of PrP^{C} is still unknown, however many different molecules interacting with PrP^{C} have been determined (molecules described are summarized in table 1), some of which provide clues about PrP^{C} biology and function. In addition several studies have shown PrP capable of binding/interacting with nucleic acids (both DNA and RNA) and most cellular processes occur under RNA supervision (reviewed in [15]). Even though these findings are

intriguing they were obtained either *in vitro* or *ex vivo* and until it is clear whether prion propagation occurs extracellularly or intracellularly, it remains to be seen whether *in vivo* nucleic acids and PrP^C are functional interaction partners. Overall it seems that PrP^C can interact with a wide variety of molecules. Molecules interacting with PrP^C can roughly be categorized as molecules (mainly negatively charged, i.e. DNA, RNA, proteoglycans) involved in cellular protein biology or partner-proteins in signalling cascades. These cascades in turn usually influence cell-survival and/or cell-differentiation pathways.

Prion protein interaction partners.

The ultimate aim when devising new therapeutic strategies is to end up with a cure which allows for retention or restoration of normal physiological functionality of the target. In the case of prion disease, it may prove crucial to elucidate the physiological function of PrP^C and in particular its interactions and interaction-partners. This may further provide information on where, when and how PrP^C can be recruited by PrP^{Sc} and subsequently converted. As a result, this knowledge may give rise to new modes and targets for intervention. At first glance, PrP^C seems to be quite promiscuous and interacts with many different proteins (Table 1).

PrP biology related interaction partners. Part of the normal biology of cellular prion protein is internalization, degradation and/or recycling hereof. Therefore it is not surprising that some of the discovered protein partners of PrP^C are involved in binding/internalization of PrP^C. Both PrP^C and the 37kDa/67kDa -laminin receptor (LRP/LR) are abundantly present in detergent insoluble lipid rafts. LRP/LR was shown to (directly) interact with PrP^C [16], and acts as a cell-surface receptor for PrP^C [17] as well as a receptor for PrP^{Sc} [18]. PrP^C and LRP/LR partly interact through heparan sulfate proteoglycan (HSPG)-dependent interaction sites [19] confirming the role of heparin-like molecules (glycosaminoglycans or GAGs) in prion biology. Internalization by LRP/LR accounts only for 20 to 50% of membrane bound recombinant-PrP^C uptake and therefore there must be another mode of internalization of PrP^C. This other mode of internalization most likely depends on clathrin-mediated endocytosis of PrP^C [20-22]. However, PrP^C is GPI anchored and can not interact directly with the clathrin endocytic machinery on the cytoplasmic face of the plasma membrane, and this gap is likely bridged by the transmembrane low-density lipoprotein receptor-related protein 1 (LRP1) [23]. Recently this notion was corroborated by showing LRP1 to be a cofactor during biosynthetic and endocytic transport [24].

Table 1. ***Summary of discussed PrP interaction partners.***

Interaction molecule	involved in	reference
LRP/LR	endocytosis of PrP^{C} / PrP^{Sc}	[16,17] / [18,155]
HSPG	aids in PrP uptake by LRP/LR	[19,156]
clathrin	endocytosis	[21,22,157]
LRP1	Intermediate in PrP^{C}-clathrin interaction	[23]
caveolin	activation fyn kinase and subsequent ERK1/2[a] activation modulation GPRC	[34,158-160]
ZAP-70	activation fyn kinase resulting in ERK1/2[a] activation	[35]
NCAM	activation fyn kinase resulting in ERK1/2[a] activation	[36]
lamanin	ERK1/2[a] activation	[37,161]
STI1	neuroprotection / regulation PrP-dependant SOD-activity	[27] / [162]
	(indirect activation of ERK1/2 via laminin?)	[27]

[a] *ERK1/2 signalling cascade is implicated of influence on redox equilibrium, cell homeostatis, cell proliferation -and differentiation*

PrP function related interaction partners. Different studies utilizing various research methodologies have directly or indirectly identified many different (possible) functional interaction partners of PrP^{C}, even though the physiological relevance is not always clear. Even though the exact physiological function of PrP^{C} remains elusive, several candidates for elucidating the physiological function of PrP^{C} emerge. PrP^{C} could have cytoprotective activity in neurons that correlates with the protective effect against apoptosis-inducing stimuli by over-expression of PrP [25,26]. Stress inducible STI1 is a specific PrP^{C} ligand that promotes neuroprotection of retinal neurons through cAMP-dependent protein kinase A [27]. The abundant expression and co-localization of both PrP^{C} and STI1 in the hippocampus *in situ*, indicates that such interaction *in vivo* is plausible. Specific interaction between STI1 and PrP^{C} (dependant on PrP^{C} binding domain of STI1) results in both neuritogenesis in wild-type neurons and neuroprotection through distinct signalling pathways [28]. A recent study revealed that STI1 triggers PrP^{C} sequestration, which is critical for activation of extracellular signal-regulated kinase ERK1/2 but not protein kinase A (PKA) [29]. The authors hypothesise that STI1 and PrP^{C} bind with each other at the cell-membrane mediating signalling by PKA whilst simultaneously triggering PrP^{C} endocytosis, which may lead to separate interactions (perhaps involving

LRP1) resulting in ERK1/2 activation. Furthermore, correlation between (specific) STI1-PrP^C interaction and memory functionality has been shown [30] and STI1-PrP^C interaction influences neuroprotective [31] and memory function [32,33] strengthening the notion that STI1 is a very likely interaction partner in PrP^C functionality.

Several studies directly or indirectly implicate interaction between PrP^C, tyrosine kinase Fyn and/or the extracellular signal-regulated kinases ERK1/2 as part of a signalling pathway involved in homeostasis [34], T-cell activation [35], nervous system development [36] and consolidation of fear memory [37]. Whether interaction between PrP^C and STI1 as well as the (in)direct interaction between PrP^C and tyrosine kinase Fyn and/or ERK1/2 are all part of the same signalling pathway involved in cell-survival and/or differentiation, remains to be elucidated. The involvement of PrP^C in this type of signalling cascade has been strengthened by microarray analysis aimed at identifying human prion protein cellular interactors [38] and revealed a significant relationship with AKT (cellular signalling/survival protein kinases, inhibiting apoptosis), JNK (stress stimuli responsive MAPK) and MAPK (extracellular stimuli or mitogen-activated protein kinases) signalling pathways. Intriguingly, it has been recently shown that PrP^C processing is related to signal transduction, with interdependence between the primary PrP^C sequence and the cell membrane [39]. In this study, increased basal cleavage was associated with lower basal ERK phosphorylation, but increased cleavage due to membrane perturbation (in the presence of copper) resulted in increased signal transduction through MAPK intermediates.

Therefore the emerging but still incomplete picture is that PrP^C is part of an intricate and complex macromolecular complex in which it plays a pivotal role (perhaps as a signalling receptor) in deciding the faith (death or differentiation) of a particular cell, depending on the interactions that occur with PrP^C. Part of these processes may involve binding of copper, just as copper is involved in the uptake of PrP^C as part of the regular life-cycle of PrP^C. It is obvious that depending on the origin of a cell, or system used in further studying interactions of PrP^C, the particular 'constituents' of this macromolecular complex may vary.

Prion protein conversion.

The protein only hypothesis hinges on the notion that disease associated PrP^{Sc} can force its conformation on PrP^{C}, better known as conversion. In order for PrP^{C} to be refolded to the disease associated conformation, monomers and/or dimers need to partially unfold and (likely) form a trimer before completely refolding into the PrP^{Sc} conformation [40]. Two mechanisms explaining the infectious character by which PrP^{Sc} induces conversion of PrP^{C} have been proposed of which the nucleation-dependant polymerization model [41] correlates best with data obtained by *in vitro* conversion. In this model a polymer of PrP^{Sc} recruits a PrP^{C} molecule (at the polymerisation- or nucleation site) after which the tertiary structure of PrP^{C} is refolded to that of PrP^{Sc}. The now 'elongated' PrP^{Sc} polymer can recruit a new PrP^{C}.

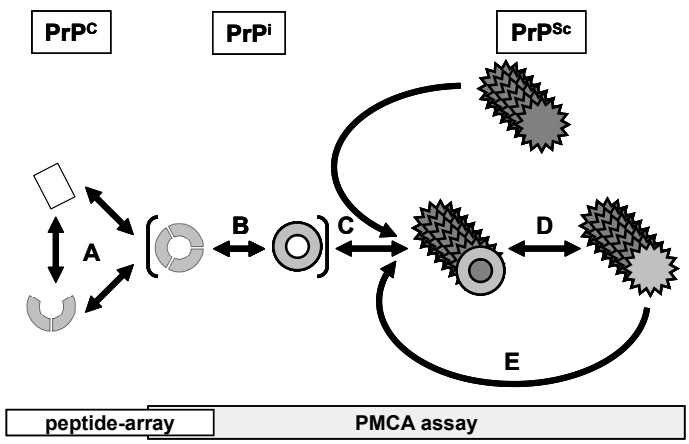


Figure 1. ***Schematic representation of likely steps involved in prion protein conversion.*** *The conversion process of prion protein is composed of different steps in which the intermediate forms of PrP are in equilibrium with each other. Based on results with CD spectroscopy, analytical ultracentrifugation, and chemical cross-linking cd-spectrum of the prion protein fibril precursor state [40], solution-state NMR spectroscopy to monitoring the oligomerization process of a β-sheet-rich (soluble) isoform of PrP [47] as well as models of fibril formation [43,44] the following schematic was devised: either two PrP^{C} molecules first form a dimer (A) after which another PrP^{C} molecule is added to form a trimer (B) and/or three PrP^{C} molecules are assembled in a trimer at once (C). This trimer is subsequently partially unfolded/refolded into an intermediate isoform of PrP (PrP^{i}, D) which can bind to the conversion seed (PrP^{Sc}, E) after which the intermediate is further adapted to the tertiary structure of PrP^{Sc} (F). The newly elongated PrP^{Sc} in turn can act as a seed for further conversion of PrP (G). Monomers, dimers and trimers probably exist in equilibrium with each other, whereas as soon as PrP is bound to PrP^{Sc} the equilibrium is skewed towards the following state.*

Unfortunately this model does not represent the intrinsic complexity of the conversion process. The conversion process most likely starts with multimerisation of PrP^{C} and is followed by a succession of distinct steps (Fig.1). Electron crystallography was used to characterize the structure of two infectious variants of the prion protein [42]. Comparison of these two variants resulted in a model featuring β-helices, which was further refined by studying 119 all-β-folds observed in globular proteins [43]. The model features left-handed β-helical folded PrP that readily forms trimers, providing a natural template for a trimeric model of PrP^{Sc} and another (similar) β-helical model was proposed, which largely explained species and strain-specificity [44]. In both models oligomerisation/ trimerisation of PrP^{C} precedes initiation of conversion as depicted in Fig. 1A, implying an important role for PrP^{C} self-interaction in the conversion processes. Additionally, study of the amyloid-forming pathway has revealed a pre-amyloid state containing partially unfolded monomers and dimers [40,45-48]. Whether the folding intermediate of PrP (PrP^{i}) is merely the partially unfolded state (monomers and dimers) of PrP^{C} or whether it is a pre-formed trimer before further structural rearrangement towards PrP^{Sc} occurs (Fig.1, B) remains to be elucidated. Refolding (conversion) of PrP to the disease associated state of PrP is initiated by recruitment of PrP to PrP^{Sc} (Fig.1C), after which PrP is (further) rearranged to adopt the tertiary structure of the PrP^{Sc} seed (Fig.1D). The elongated PrP^{Sc} is in turn capable to recruit and convert further PrP (Fig.1E). Even though little is known on the prevalence of oligomers (polymers) *in vivo*, fractionation studies performed on PrP^{Sc} aggregates in order to elucidate what size oligomer are the most infectious prion particle [49], confirms the presence of various oligomers *in vivo*. This study showed that oligomers containing 14-28 PrP molecules were the most efficient initiators of conversion and that an infectious particle contains at least 5 PrP molecules in order to initiate conversion.

Development of a cell-free *in vitro* conversion assay [50] provided an experimental model to investigate PrP^{C} to PrP^{Sc} conversion, in which PrP^{C} can be specifically converted *in vitro* to a protease resistant state (PrP^{res}), only in the presence of PrP^{Sc} with at least some native conformation. Further investigation demonstrated that conversion occurs by two kinetically distinct steps: first binding of PrP^{C} to the PrP^{Sc} (or PrP^{res}) oligomers and second conversion of bound PrP^{C} into the PrP^{res} conformation (reviewed in [51]). Even though these experiments provide ample proof for the protein-only theory, the yield of PrP^{res} in these studies was too low for detection in *in vivo* infectivity tests (because of the required surplus of purified PrP^{Sc} necessary to initiate conversion). Nonetheless, the cell-free conversion reaction is highly specific, reproducing at least certain elements of the species-barrier and strain specificity (reviewed in [51]). Using the cell-free *in vitro* system mimicking TSE amplification, a molecular assessment of the capacity of PrP^{Sc}

from scrapie affected sheep (PrP^{Sc}), BSE affected bovines (PrP^{BSE}), CWD affected deer (PrP^{CWD}) and vCJD affected humans to convert PrP^{C} of the same species or between species was made [52]. Discrete conversion products were observed in conversion reactions of human PrP^{C} induced with PrP^{Sc}, PrP^{BSE} or PrP^{CWD}. However, despite the low efficiency, the fact that conversion occurred, does suggest that human exposure to scrapie, BSE or CWD may potentially result in pathogenic conversion to PrP^{res}.

Currently, an alternative *in vitro* conversion assay has come into use, the very sensitive protein misfolding cyclic amplification (PMCA) assay.The PMCA reaction takes place in the presence of detergent and uses crude brain extracts instead of purified PrP^{Sc} material and PrP^{C} from cell cultrues. This system of conversion is supposed to mimic physiological conditions more closely (reviewed in [53]).This is corroborated by studies confirming the generation of infectivity after *in vitro* propagation by PMCA [54,55], showing that prion strain-specific differences in biochemical and clinical features were maintained *in vivo*. The PMCA assay was utilized to ascertain which components are (minimally) needed for successful *in vitro* propagation of PrP^{Sc} molecules, which showed that next to highly purified PrP^{C} (still containing unsaturated 20-carbon fatty acids) accessory polyanion molecules are required [56]. Additionally these experiments showed that *de novo* PrP^{Sc} molecules could be formed in the absence of preexisting prions, which in turn proved infectious (causing scrapie in hamsters) and transmissible upon second passage. Altogether this raises the possibility that endogenous polyanionic cofactors may participate in *in vivo* prion propagation. Several negatively charged macromolecules may (in concordance with previous studies, as discussed by [56]) be a cofactor in prion propagation including nucleic acids[237-244], glycosaminglycans, phospholipid-rich membranes and chaperone proteins.

Interestingly, PrP^{C} can also be refolded into different stable (non-native) structures with high β-sheet content [57,58] *in vitro*. Further investigation of one of these structures, the β-oligomer (β^{0}, [57]), revealed the existence of a monomeric intermediate retaining most of the original α-helical structure of PrP^{C} and which exists in a molten globular state [47]. These findings show that intermediate states of PrP^{C} can exist (as suggested in Fig.1), and these intermediates may be of interest in elucidating the molecular processes underlying or preceding conversion.

PrPC conversion assays provide an excellent in vitro model in which relative amounts of PK resistant PrP (PrPres) reflect important biological aspects of TSEs at the molecular level. Even though these assays (in combination with in vitro refolding studies) have improved the understanding of the processes involved in prion protein conversion,

the exact molecular mechanisms underlying these processes remain elusive. The emergence of variant Creutzfeldt-Jakob disease after the BSE crisis substantiates unrelenting vigilance. Continued development of current techniques and therapeutic strategies is necessary, as well as further investigation of the physiological function, interaction(-domains) of the prion protein and the role of cofactors, which in turn may lead to novel therapeutic strategies, possibly in which PrPC functionality is retained.

Prion Protein Interaction Domains.

The molecular mechanisms underlying the conversion process are determined by similarities as well as strain dependent variations in the PrP structure. Selective self-interaction between PrP molecules is the most probable basis for initiation of these processes (Figure 1, A+B), potentially influenced by chaperone molecules. The formation of PrP^{Sc} aggregates probably requires self-interactions of PrP^{C} molecules as well as with PrP^{Sc} [43,44]. Thus binding and conformational changes are essential events in this conversion process.

Determining PrP interaction domains. Differences in susceptibility of sheep to scrapie and transmissibility of scrapie in sheep can largely be explained at the molecular level by the effects of single polymorphisms (in Prn-P) in PrP^{C} or PrP^{Sc} on PrP conversion [59-62]. However we previously showed that PrP^{C} readily binds to PrP^{Sc} and that this initial binding is PrP specific and unaffected by disease associated polymorphisms [63]. Therefore a peptide-array based on linear PrP sequences comprising the complete PrP sequence was utilized to determine which residues of the prion protein are capable of interacting with PrP^{C} [64]. Incubation of mature PrP on the peptide-array resulted in a reproducible PrP binding pattern (Fig.2) comprising two high binding areas and two low binding areas. Analysis of this binding pattern allowed determination of several interaction domains within PrP^{C}.

Within the N-terminal tail of PrP (roughly aa 15-105, amino acids numbering of ovine PrP used throughout) three interaction domains were identified; a domain covering most of the glycosaminoglycan motif KKRKP (Fig.2 domain 1, re-evaluated because of follow-up study results [65]), a domain covering the octarepeat sequence [PxGG], which is repeated five times (Fig.2, repeats A-E) and a domain (Fig.2, domain 2) directly adjacent to the limiting region containing strain and species dependant variable sites for proteinase K trimming (Fig.2, arrows) of PrP^{Sc} [66,67].

The C-terminal region of PrP^C (roughly aa 105-225) contains four different binding domains, of which three are situated in the C-terminal core (roughly aa 105-180), whereas only one was found at the C-terminus (roughly aa 180-225). These domains are situated in the secondary structure of PrP as follows (Fig.2): domain 3 between β-sheet 1 and α-helix 1, and domain 4 with two aa repeats respectively within α-helix 1 (4^a) and within β-sheet 2 (4^b). Domain 5 is located in the core within α-helix 2 and domain 6 at the C-terminus directly adjescent to α-helix 3. Both these domains are previously unidentified domains. Interestingly, all binding domains were part of epitopes recognized by antibodies that specifically immuno-precipitate infectivity [68-71] or antibodies that prevent scrapie infection either *in vitro* [72,72,73,73,74,74,75,75,76,76] or *in vivo* [77], except for domain 5, attributing validity to the binding role of the determined domains.

Determining the role interaction domains play. Based on the interaction domains described above [64] and properties reported in literature, six ovine PrP regions were selected and peptides from these regions used to assess the effect of these regions on two

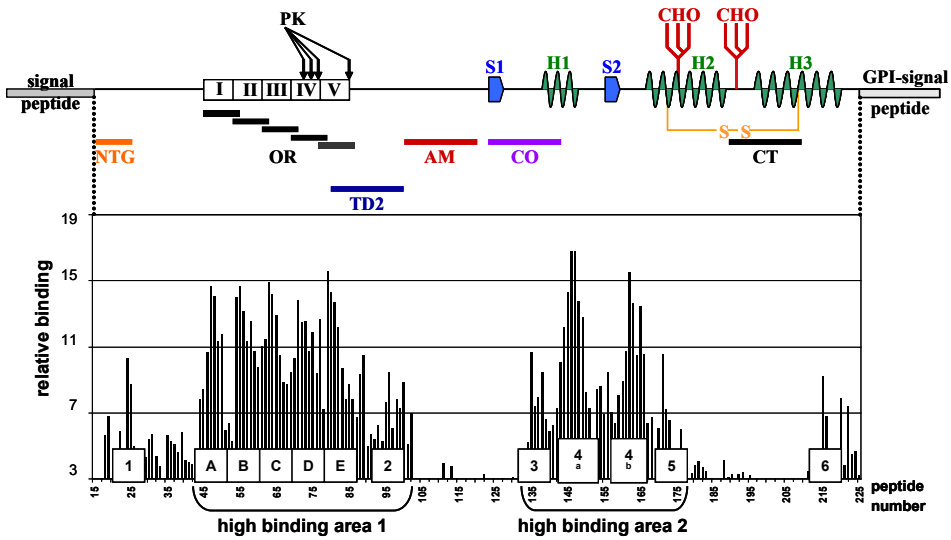


Figure 2. ***PrP^C secondary structures and relative peptide positions versus peptide-array binding pattern and binding domain positions.*** *Schematic representation of PrP^C (adapted from Fig.5 [64]) depicting signal sequences, β-sheets (S1, S2), α-helices (H1, H2, H3), disulfide bridge site (S-S), glycosylation sites (CHO) of PrP^C and the relative positions of the peptides used in this study. The bar graph represents the previously determined peptide-array binding pattern [64][64] and the relative positions of the determined binding domains: 1 [27-RPKPGGG-33], A-E octarepeat motif [P(H)GG], 2 [102-WNK-104], 3 [140-PLIHFGNDY-148], 4^a [152-YYR-154], 4^b [165-YYR-167], 5 [177-NFV-179] and 6 [225-SQAY-228]. All numbering used is for sheep PrP.*

processes of importance in prion disease development; self-interaction and conversion [65]. To this end effects of the selected peptides in both the peptide-array and the protein misfolding cyclic amplification (PMCA) assay were studied. This study shows that the previously determined self-interaction domains of PrP^{C} are of importance at several different phases in the conversion reaction. Our data corroborate suggestions that the region including interaction domain 3 (Fig.2) is involved in species specificity and susceptibility determinants of PrP^{C}. Our data further suggest that the octarepeats and the glycosaminoglycan binding -and the amyloidogenic motif can indirectly affect the processes underlying PrP conversion. The octarepeats seemingly stabilize PrP^{C}-PrP^{Sc} interaction (Fig.1, C), whereas the glycosaminoglycan binding and amyloidogenic motif seem to induce structural (tertiary structure) changes in PrP^{C} (Fig.1, B) facilitating subsequent conversion of PrP. Direct involvement in (di or-) trimerisation of PrP^{C} (Fig.1, A) is implicated for the two binding domains 2 and 6, which precedes partial unfolding or refolding into PrP^{i}, binding to PrP^{Sc} and subsequent conversion. Therefore these two domains may prove prime targets for development of new therapeutic strategies. These results further emphasize the importance of the stability of the PrP^{C}-PrP^{C} and PrP^{C}-PrP^{Sc} interactions in PrP conversion, which probably is at the basis of the effects of disease associated mutations, as well as the species-barrier. Increased understanding of the processes involved in PrP conversion may lead to the conception of therapeutic strategies with the possibility to leave the physiological function of the prion protein unaffected.

Therapeutic strategies in prion disease.

Even though the physiological function of the prion protein, as well as the exact molecular processes underlying conversion of PrP^{C} by PrP^{Sc} remains elusive, different therapeutic compounds and strategies are (being) investigated (summarized in table 2). Nowadays the protein-only theory has become widely accepted and stipulates that PrP^{Sc} represents the infectious entity in prion disease and therefore current therapeutic strategies are primarily aimed at preventing conversion of PrP^{C} by PrP^{Sc}. Different strategies can be applied in order to prevent interaction of PrP^{C} with PrP^{Sc} and so averting subsequent conversion (and PrP^{Sc} accumulation). This can be accomplished by restricting availability of PrP^{C} (PrP^{C} depletion by RNAi, conversion resistant PrP^{C} isoforms, blocking peptides) for conversion by PrP^{Sc}. Alternatively, PrP^{Sc} may be prevented from migrating to the CNS or measures may be taken to increase/aid clearance of PrP^{Sc} (branched polyamines, β-breaker peptides, and calpain dependant

cleavage). Many approaches to date have been aimed at interfering with binding of PrP^{C} to PrP^{Sc} (immuno-therapy, heparin mimetics, quinacrine). Many compounds and strategies have been tested, however only the most promising are discussed below.

PrP^{C} based strategies.

The most straight forward method preventing conversion of PrP^{C} is making PrP^{C} unavailable for conversion. By abolishing the presence of PrP^{C}, there is no base from which to form new PrP^{Sc}. Ideally this would be a reversible process, so that the physiological function of PrP^{C} may be restored after treatment.

PrP^{C} depletion. Part of studies into the physiological function of PrP^{C}, was the transgenic modification of mice resulting in PrP knockout mice ($PrP^{0/0}$). Using these $PrP^{0/0}$ mice it was shown that mice lacking PrP^{C} are not susceptible to prion disease [78]. However, resorting to transgenic modification as a therapeutic strategy is quite drastic. More incouragingly, a mouse model in which PrP^{C} expression can be eliminated after scrapie infection has been established showed reversal of early neurodegenerative changes caused by prion infection and long-term protection against neuronal loss despite continued prion replication and PrP^{Sc} deposition [79]. Unfortunately PrP^{C} depletion in this model is also permanent and it is unclear whether the loss of PrP^{C} functionality has a negative (long term) effect. Discovery of RNA interference has led to feasible means of depleting PrP^{C} without having to resort to transgenic modification. In order to study down regulation of PrP^{C} expression we cloned and tested antisense (reverse complement of *PRNP* ORF) constructs of ovine PrP^{C} in a Chinese hamster ovary (CHO) cell line stably expressing different variants of ovine PrP^{C} [80]. Transcription of these antisense constructs produces mRNA that is complementary (negative strand) to 'normal' PrP^{C} mRNA. The resulting formation of double stranded RNA activates a conserved mechanism in which double stranded RNA is naturally cleaved and recycled by the cell, preventing translation and expression of PrP^{C} (Fig.3, B). We were able to reduce PrP^{C} expression to 50% of normal when co-expressing our antisense constructs in stably transfected CHO cells expressing different variants of ovine PrP^{C}, and reduction seems irrespective of genotype of either PrP^{C} or antisense construct (unpublished data). Prompt advances in RNA interference strategies resulted in development of small interfering RNA (siRNA) and its variant short hairpin RNA (shRNA) which will activate the conserved RNA interference mechanism. By using small interfering RNAs (siRNA), the steady state amount of *PRNP* mRNA expression, and thus PrP^{C} expression in cultured cells was down-regulated and effective in preventing PrP^{res} formation in scrapie infected neuroblastoma cells [81]. Short hairpin RNAs (shRNAs) have been developed in a lentiviral delivery system and was shown

Table 2. ***Overview of therapeutic compounds/strategies.***

therapeutic	*In vitro*	*In vivo*	Human 'trials'	Therapeutic usability	References
PrP^{c} based strategies					
PrP^{c} depletion					
siRNA	pos.	n.d.	n.d.	t.b.d.	[81,163]
shRNA	pos.	pos.	n.d.	t.b.d.	[82,83]
Heterelogous PrP and PrP Peptides					
Heterelogous PrP	pos.	pos.	n.d.	t.b.d.	[86,89,95,164]
Amyloidogenic motif peptide	pos.	n.d.	n.d.	unlikely	[96,165]
Strategies targeting PrP^{Sc}					
Preventing PrP^{Sc} migrating to CNS					
LT beta R-Ig	n.d.	pos.	n.d.	t.b.d.	[103,104,166]
Branched (dendritic) polyamines	pos.	pos.	n.d.	prophylactic / t.b.d.	[105,106]
ß-breaker peptides	pos.	pos.	n.d.	unlikely	[107,109,121]
Calpain dependant cleavage of PrP^{Sc}					
Calpain inhibitors (quinacrine)	pos.	n.d.	n.d.	t.b.d.	[113]
Strategies aimed at disrupting PrP^{c} - PrP^{Sc} interaction					
Immuno-therapy					
Fabs	pos.	n.d.	n.d.	unlikely	[75]
Active immunization	n.d.	pos.	n.d.	t.b.d.	[121-123]
Passive immunization	n.d.	pos.	n.d.	t.b.d.	[120,124]

n.d. = not done

t.b.d. = to be determined

c.u. = currently underway

Table 2, continued.

therapeutic	*In vitro*	*In vivo*	Human 'trials'	Therapeutic usability	References
Polyanions					
Dextran sulfate	n.d.	pos.	n.d.	prophylactic	[128,167,168]
Pentosan polysulfate	pos.	pos.	mixed	mixed results	[18,128,130-133]
Heparan mimetics	pos.	mixed	n.d.	unclear	[18,129,130]
Sulfonated dyes					
Congo red / analogues	pos. / pos.	pos. / pos.	n.d.	no / prophylactic	[18,128,129]
Suramin / analogues	pos. / pos.	pos. / n.d.	n.d.	prophylactic / t.b.d.	[169,170]
Cyclic tetrapyrrols					
Poryphyrines / phtalocyanines	pos.	pos.	n.d.	prophylactic	[140-142,171]
acridine and phenothiazine derivatives					
Quinacrine / chlorpromazine	pos.	neg.	neg. / n.d.	no / unlikely	[142-153,172]
Quinine / biquinoline	pos.	some	n.d.	unlikely	[147]
Tetracylines					
Tetracycline, doxycicline	pos.	pos.	c.u.	possibly	[154]

to interfere with PrP^{C} expression and subsequent PrP^{res} formation in tissue culture and was capable of down-regulating PrP^{C} expression at the injection site in mice, prolonging the survival of these scrapie infected mice [82]. However, the authors themselves state that effective therapeutic use of lentiviral shRNA vectors require designing shRNA that achieve efficient and selective knock down and to develop techniques to efficiently transduce the target cells, which currently requires high-titer vector preparation. Injection into the hippocampus of lentivirus expressing shRNAs in order to treat mice with established (pre-clinical) prion disease prevented the first behavioural deficits associated with early pathology of the hippocampus CA1 region [83]. This study also shows that lentivirus does not spread beyond the site of infection, and, in order for more extensive neuroprotection and longer survival, more widespread transduction, enhanced mechanistic delivery, and/or even focal targeting is needed. Lentiviruses efficiently integrate their genome into neuronal (non-dividing) cells [84,85], and if shRNAs were capable of completely clearing PrP^{Sc} then it would be advantageous if knockdown of PrP^{C} could be reversed and PrP^{C} functionality restored, which makes genomic integration of shRNA expressing lentivius undesirable. In addition, lentiviral shRNA vectors do not spread in neuronal tissues, restricting the positive effects to the site of injection, whilst TSEs are characterized by disseminated degeneration of neuronal cells throughout the brain. It may therefore be pertinent to look for other delivery vectors of shRNAs that can overcome the shortcomings of the lentiviral delivery system in order to obtain a viable therapeutic strategy using shRNAs. A possible candidate delivery vector may be pseudorabies virus, which has a known tropism for neuronal tissues. We have been able to clone the PrP^{C} open reading frame into pseudorabies virus (described below) resulting in (transient) expression of PrP^{C} in tissue culture.

Heterologous PrP and PrP-peptides. Many reports have clearly demonstrated that compatibility of PrP^{C} and PrP^{Sc} is necessary for efficient conversion of PrP^{C} by a particular PrP^{Sc} strain. Incompatibility can be a result of differences in prion protein between species [86] or specific amino acid substitutions [87,88]. *In vitro* studies indicate that incompatible PrP^{C} not only resists conversion (Fig.3C, a) but is also capable of blocking conversion of compatible conversion sensitive PrP^{C} molecules present. Heterozygosity is essentially enough to inhibit conversion [86,89] of conversion sensitive PrP^{C}. Expression of incompatible PrP^{C} may therefore be a viable therapeutic strategy in prion disease, for mutations in PrP^{C} do not affect the initial binding to PrP^{Sc} [63,90,90], especially because expressing full length conversion resistant PrP^{C} will most likely conserve PrP^{C} functionality. An example of prophylactic use of conversion resistant PrP^{C} is the preferential breeding of resistant phenotypes in flocks of sheep. Especially in the Netherlands this breeding-program has been widely and effectively implemented. A note

of caution has to be made however; cases of natural, atypical and experimental scrapie infection in sheep with a resistant PrP genotype have been reported [91-94]. Treatment of transgenic mice with PrPQ167R, a lentiviral expressed mouse PrP^{C} containing the mutation equivalent to dominant negative sheep Q171R and human E217K mutations, increased survival times of the treated mice [95]. Prolongation of survival time was still achieved when treatment was started at a late state of infection. However, the treatment requires chronic injection through a guide cannula directly to the brain and reduction of spongiosis was only at the cannula side of the brain. These results are intriguing and promising, but in order for this strategy to be viable, a delivery system to get conversion resistant PrP^{C} continuously expressed in all affected neuronal tissues needs to be devised. A probable delivery system would be use of a viral delivery system which can (naturally) cross the blood-brain barrier and has a tropism for neuronal tissues. To this end we have manipulated known virulence genes of the pseudorabies virus, in order to attenuate the virus and at the same time have expression of conversion resistant PrP^{C}. We were able to get recombinant virus expressing PrP^{C} in tissue culture (unpublished data), however the applicability of this strategy needs to be investigated further.

Next to full length conversion-resistant PrP^{C}, different peptides have been tested for their capacity to block conversion. Specific peptides containing a conserved aa motif in PrP^{C} were capable of inhibiting PrP^{res} formation in both a cell free *in vitro* conversion system as well as cell culture [96]. However, peptides have several disadvantages when considering them for therapeutic use; peptides are sensitive to proteolytic degradation and crossing the blood brain barrier is very ineffective.

Strategies targeting PrP^{Sc}.

Averting PrP^{C} to PrP^{Sc} conversion can also be accomplished by preventing PrP^{Sc} from reaching the neuronal tissues, thereby preventing disease development. Another necessity is development of therapeutic strategies in which already formed PrP^{Sc} can be degraded or cleaved. Some promising studies have been done, however the applicability as a therapeutic needs to be assessed.

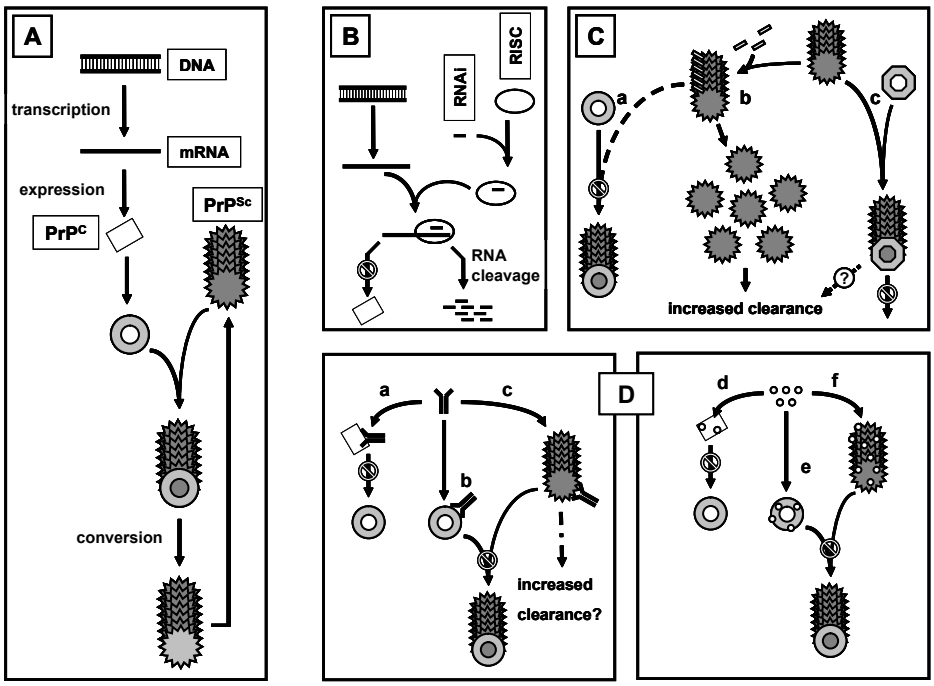
Prevention of PrP^{Sc} migration to CNS. After oral (or via lesions in skin [97]) exposure to PrP^{Sc}, the infectious agent needs to be transferred from the gastrointestinal tract to the CNS. Several studies have suggested that PrP^{Sc} first accumulates in B-cell follicels of the follicular dendritic cells (FDCs) within the gut associated lymphoid tissues (GALT) [12,98,99] with a critical role for the Peyers patches in PrP^{Sc} uptake, from where neuronal invasion occurs. Several cells have been suggested to mediate PrP^{Sc} uptake and transport

to the FDCs. An *in vitro* study implicated membranous epithelial cells (M) cells in trans-epithelial uptake of PrP^{Sc} [100], however intestinal epithelial cells may also be capable to transcytose PrP^{Sc} [101]. Another recent *in vitro* study demonstrated that migratory $CD11c^{+}$ cells, most likely dendritic cells, play a key role in the translocation of the scrapie agent from the gut lumen to the GALT from which neuronal invasion subsequently occurs [102]. Neutralization of the LTb-R pathway has been shown to prevent maturation of FDCs and subsequent scrapie neuroinvasion [103,104] and this approach was most successful when treatment was administered prior to scrapie inoculation. These findings were corroborated by a study in which FDCs were transiently dedifferentiated prior to skin inoculation or 14 days post skin inoculation [97]. However, even though this approach may be viable in prion disease treatment, this may only be prophylactic.

Branched (Dendritic) polyamines. The first clue that branched polyamines possess anti-prion properties was the discovery that SuperFect decreased both pre-existing PrP^{Sc} and prevented formation of *de novo* PrP^{Sc}. The active compounds were identified as the dendritic polymers polypropyleneimine (PPI) generation 4.0, polyethyleneimine and polyamidoamide generation 4.0. Further investigation showed that treatment of scrapie infected cell line N2a (ScN2a) with PPI generation 4.0 not only cured these cells but also eradicated prion infectivity in a mouse bioassay [105]. *In vitro* the branched polyamines were shown to disaggregate prion rods, reducing the beta-sheet content of PrP 27-30 making it susceptible to proteolysis. However, the susceptibility to proteolysis *in vitro* was strain dependant; PrP^{Sc} from BSE was susceptible whereas PrP^{Sc} form natural scrapie was resistant. Another promising novel class of dendritic polyamines are phosphorus-containing dendrimers, which have improved bioavailability and decreased toxicity compared to the polyamines reported by Supattapone and are potentially relevant in post-exposure prophylactic prion therapy [106].

Taken together it seems that branched polyamines are capable of interacting with PrP^{Sc}, resulting in blocking of de novo PrP^{Sc} formation (Fig.3D, d) as well as increased PrP^{Sc} clearance. The exact mechanism in which this compound increases PrP^{Sc} clearance is not understood, however further investigation hereof may provide clues to how clearance is stimulated.

Figure 3 (following page). ***Schematic representation of steps involved in prion protein expression, conversion and possible modes of interference.*** *Several steps are involved in expression of host encoded PrP^{C}, which once expressed may be recruited by PrP^{Sc} and subsequently converted [A]; first the gene encoding Prnp is transcribed, producing messenger RNA (mRNA) encoding for PrP^{C}. This mRNA is transported from the nucleus to the cytosol where the ribosomal complex attaches and*



translates the mRNA to produce PrP^C (expression) after which some posttranslational modifications are made before translocation onto the extracellular membrane. After PrP^C is expressed on the cell membrane it can form trimers by self-interaction and partially un/refold into an intermediate isoform of PrP (PrP^i). In turn PrP^i may be recruited by PrP^{Sc} and converted into PrP^{Sc}. Several modes of disrupting conversion of PrP^C into PrP^{Sc} are possible: By using RNAi the natural mechanism removing double stranded RNA can be triggered [B], removing mRNA and preventing expression of PrP^C. Instead of preventing PrP^C expression, compounds or peptides can be added that interact with PrP^{Sc}, resulting in dissemination of the PrP^{Sc} seed, preventing recruitment of PrP^C [C, a] and/or aiding in enhanced clearance of PrP^{Sc} [C, b]. Alternatively, heterelogous conversion resistant PrP^C can be expressed, which is recruited by PrP^{Sc}, stopping conversion and rendering the bound seed useless and possibly allowing for increased clearance of PrP^{Sc} [C, c]. Finally, the interaction between PrP^C and PrP^{Sc} can be interrupted [D]. This can be done by passive immunization, in which antibody (fragments) bind to either the PrP^C monomer [D, a], the PrP trimer or PrP^i [D, b] or to the PrP^{Sc} seed [D, c]. The latter may opsonise the PrP^{Sc} seed for increased clearance. In active immunization, the antibodies most likely bind either the PrP^C monomer [D, a] and/or the PrP trimer or PrP^i [D, b]. However, binding of the PrP^{Sc} seed [D, c] can not be completely ruled out. Antibody binding (sterically) hinders interaction between PrP^C, and PrP^{Sc} is preventing PrP^C recruitment and conversion by PrP^{Sc}. Alternatively, chemical compounds may be administered that bind monomeric PrP^C [D, d], the PrP trimer or PrP^i [D, e] or to the PrP^{Sc} seed [D, f], similarly preventing PrP^C and PrP^{Sc} interaction.

β-sheet breaker peptides. Different from peptides used for active immunization or PrP-derived peptides determined to block cell-free *in vitro* conversion, was the effect of peptides designed to specifically interact with the PrP region containing the β-sheets (β-breaker peptides) [107-109]. Several peptides were initially designed with one peptide capable of β-sheet structure disruption, reversing protease resistance of PrP^{Sc}, preventing PrP^{res} formation in scrapie infected tissue culture cells and increasing survival of mice inoculated with PrP^{Sc} pre-incubated with this peptide (Fig.3C, b). As with the peptides capable of blocking cell-free *in vitro* conversion these peptides have several disadvantages when considering them for therapeutic use; peptides are sensitive to proteolytic degradation and crossing the blood brain barrier is very ineffective.

Calpain dependant cleavage of PrP^{Sc}. PrP^{C} and PrP^{Sc} have the same origin and are two (iso)forms of the same protein, which have distinct biochemical differences (Table 2). However, *in vivo* PrP^{C} and PrP^{Sc} are subject to diverse intracellular proteolytic processing events [110-112]. Whereas PrP^{C} is cleaved to produce a ~17kDa C-terminal fragment, PrP^{Sc} is cleaved at the same location that PK cleaves PrP^{Sc} *in vitro* into PrP27-30. Using scrapie infected mouse (SMB) cells, differences in proteolytic processing events of PrP^{Sc} and PrP^{C} were faithfully reproduced, which confirms the difference in proteolytic processing of PrP^{Sc} observed *in vivo* [113]. Stimulated by increased intracellular calcium, production of PrP^{Sc} cleavage product PrP27-30 is augmented. However, pharmacological inhibitors of calpains and overexpression of the endogenous calpain inhibitor (calpastatin) prevents the increase of PrP27-30. Therefore it is suggested that calpain-mediated endoproteolytic cleavage (modulated by Ca^{2+}) of PrP^{Sc} may be an important event in prion propagation, which seems to be corroborated by a study determining calpain as one of the cytosolic proteases contributing to the degradation of retro-translocated PrP in the cytosol when proteasome activity is compromised [114]. Furthermore, scrapie infection induces abnormalities in Ca^{2+} homeostasis [115] and treatment of human neuroblastoma cells with the neurotoxic PrP(106–126) peptide resulted in a rapid rise of intracellular calcium associated with an increase in calpain activity [115,116]. Therefore Yadavalli *et al* [113] suggested that the anti-prion effects of the acridine and phenothiazine derivatives like quinacrine (which blocks Ca^{2+} channels) may be due to decreased intracellular Ca^{2+} levels resulting in a lower calpain activity. Even though it remains unclear how exactly calpain endoproteolytic cleavage (seemingly) modulates PrP^{Sc} degradation, further investigation hereof may provide a therapeutic strategy for reducing already formed PrP^{Sc} in prion disease.

Strategies aimed at disrupting PrP^{C} - PrP^{Sc} interaction.

When PrP^{C} can not be (reversibly) down-regulated or if PrP^{Sc} reaching the neuronal tissues can not be prevented, development of disease may be stopped by interfering in the interaction between PrP^{C} (Fig.3, A+B, C) molecules or by interfering in PrP^{i}-PrP^{Sc} interaction (Fig.3, E). This may be done through passive immunization (targeting both PrP^{C} and PrP^{Sc}), by active immunization (more likely targeting PrP^{C}) or by chemical compounds that can interrupt PrP^{C} (PrP^{i}) interacting with PrP^{Sc}.

Immuno-therapy. One way of disrupting PrP^{C} to PrP^{Sc} binding and subsequent conversion, is the use of antibodies. Pre-incubation of antibodies with infectious microsomes isolated from hamster brain homogenate reduced prion titer [117] and specific antibodies were able to interrupt binding of PrP^{C} and PrP^{Sc}, preventing subsequent PrP^{res} formation in cell-free *in vitro* conversion experiments [118] (Fig.3D, a+b). Several recombinant antigen-binding antibody fragments (Fabs) specific for PrP^{C} domains were shown to prevent PrP^{res} formation and cure already present PrP^{Sc} in scrapie infected neuroblastoma cells [75] (Fig.3D, b2). Transgenic expression of the anti-PrP antibody 6H4 μ-chain protected mice from prion disease after intraperitoneal inoculation [77], which thus showed the potential of immuno-therapy in prion disease. Active immunization (stimulating the vaccinated subject to produce specific antibodies) can be achieved by vaccination with recombinant PrP, circumventing immune-tolerance to PrP in mice and prolonging survival, especially when vaccinated before exposure to scrapie [119] (Fig.3D a). Active immunization with a synthetic prion protein derived peptide also significantly prolonged survival of vaccinated mice [120,121], however peptides can be sensitive to proteolytic degradation. Two recent studies have devised a system allowing for stable expression of (partial) PrP. Firstly, full-length PrP or C-terminal sequences of PrP were expressed on retroviral particles [122] and single intravenous immunization of mice with these particles resulted in both a PrP-specific IgM and IgG response. Whether the induced antibody titres are sufficient in preventing prion disease is under investigation. Secondly, the peptide DWEDRYYRE was expressed on papillomavirus-like particles and induced high antibody titers in rabbit. This rabbit-anti-PrP serum contained high affinity antibody able to inhibit PrP^{res} formation in prion infected cells [123]. A drawback of active immunization may be that PrP^{C} functionality is impaired indefinitely and therefore passive immunization may well be the more pertinent solution. Passive immunization also proved a successful immuno-therapy strategy. Administering specific anti-PrP monoclonal antibodies directly after interperitonal inoculation of mice with scrapie resulted in prolonged survival of these mice [120]. Continued treatment of mice with antibodies after the onset of peripheral prion replication and (30 days post

infection) delayed onset of scrapie by more than 1,5 times the usual incubation period [124]. However, when treatment was started after onset of clinical symptoms, passive immunization was not effective.

All data described above do make the immunotherapeutic intervention in prion disease a strategy worth pursuing (also reviewed in [125]). Moreover a similar immunisation strategy targeted at Aβ has proved effective in reducing amyloid deposits in animal models of Alzheimer disease. However, all effective antibodies to date are only effective as prophylactics. Furthermore, *in vivo* cross-linking with PrP^{C} specific antibodies triggered rapid and extensive apoptosis in hippocampal and cerebellar neurons [126] and therefore the specific mechanisms by which successful immuno-therapy is effective need to be further determined. If immuno-therapy occurs with antibodies capable of cross-linking PrP^{C}, this may possibly lead to apoptosis depending on the amount of the antibody present. In the case of passive immunization it may be advisable to use a PrP^{Sc}-specific antibody, especially when longer periods of treatment are necessary.

Polyanions. Sulphated glycans are known to non-specifically and indirectly inhibit entry of several viruses and were first tested because prions were thought to be an unconvential virus. Examples are dextran sulphate [127], pentosan polysulfate [18,128] and heparan mimetics [18,129,130]. Polyanions were shown to be effective in protecting rodents against infections, except for heparan mimetics. A drawback of polyanions is that penetration of the blood-brain barrier is minimal, which may also explain why polyanions are only effective prophylactically. Pentosan polysulfate (PPS) showed promise as a therapeutic when administered to a vCJD patient into the cerebral ventricular system via a permanently implanted right frontal intraventricular catheter, connected to a subcutaneous programmable pump [131] to circumvent the blood-brain barrier. However, another case in which pentosan polysulfate was used as therapy in a vCJD patient at a relatively earlier stage, the treatment was to no avail [132]. A recent monitoring study of seven British patients treated with PPS showed that complications of intraventricular catheterization was frequent, that PPS was tolerated over a wide dose range and that mean survival time of these patients was prolonged [133]. However, an editorial in the same journal issue [134] does point out there are still many uncertainties and that the therapeutic usefulness of pentosan polysulfate remains unclear.

Several heparan mimetics (Congo red, porphyrins and phtalocyanins) have been identified capable of binding to PrP^{C} and preventing PrP^{Sc} production *in vitro* (Fig.3D, c+d2). Congo red was tested for its anti-prion capacity, because of its amyloid binding properties [135,136]. Even though Congo red displayed PrP^{Sc} inhibitory capability

(Fig.3D, d2), it has some practical shortcomings; it binds aspecifically to amyloid, it can only very limitedly pass the blood-brain barrier and it can be cleaved by enzymes in the mammalian gut and intestines resulting in the release of the highly carcinogenic benzidin. Therefore analogues of Congo red were designed to improve specificity, blood-brain barrier permeability and/or resistance to cleavage [137]. Interestingly curcumin (yellow pigment in tumuric) shares many properties of congo red, except for the sulphonates and benzidin, was an effective inhibitor of PrP^{Sc} formation [138]. Effectiveness of curcumin has only recently been tested *in vivo* (using the C57BL/6-mouse/scrapie strain 139A model), resulting in a significant prolonged survival time at the lower dose tested (high dose had no effect) [139]. However, it is not clear by which mechanism curcumin prolongs survival (through its anti-oxidative/anti-inflammatory properties, or by direct inhibition of prion replication, or both). Nevertheless, the low toxicity of curcumin, its capability to inhibit prion replication *in vitro* and the prolongation of survival times *in vivo* do provoke further study.

Porphyrins and phtalocyanines have structures resembling Congo red (aromatic rings, sulfonate groups) and can bind selectively and strongly with proteins resulting in a conformational change, making them interesting as inhibitors. These tetrapyrroles were tested for their inhibitory capacity and certain tetrapyrroles were found to inhibit PrP^{res} formation in a mouse scrapie-infected cell-line (ScNB) and a hamster in vitro conversion system [140][134]. When tested in hamsters three tetrapyrroles could prophylactically inhibit PrP^{Sc} formation and prolong survival (likely Fig.3D, c+d1), however later stage treatment (at onset of clinical symptoms) did not result in significantly prolonged survival rates [141], which was confirmed when tested in different mouse lines [142].

Acridine and phenothiazine derivatives. Acridine and phenothiazine derivatives are known to pass the blood brain barrier and are capable of inhibiting PrP^{res} formation in a cellular model of prion disease (Fig.3D, c+d). Several studies have shown that these compounds (which include the antimalarial and antipsychotic therapeutical drugs quinacrin and chlorpromazine) are effective inhibitors of PrP^{res} formation *in vitro* [143-147]. Of these compounds only quinine and biquinoline have shown slight efficacy in rodents when administered intraventricularly by osmotic pumps [143]. Even though quinacrine did not show anti-prion effects in intercerebrally infected rodents this drug has been used extensively as an experimental (compassionate) treatment in CJD patients [143,148-152]. Although treatment sometimes resulted in slight improvement in the condition of the patients, this improvement was only temporary and without significant delay of disease progression. One report specifically reported that quinacrine administration had an adverse effect: liver dysfunction [153]. Another study also reported liver dysfunction

after starting quinacrine treatment [148] but did not specifically link it to administration of quinacrine. And even though it has been reported that the toxic effect of quinacrine on liver function can be averted (because quinacrine dose could be lowered) by co-administration of verapamil (improves quinacrine transport over the blood-brain barrier) [152], these studies do warrant further evaluation of the toxic side-effect of quinacrine treatment and the general unsuccessful quinacrine treatment of CJD patients makes the therapeutic feasibility of quinacrine doubtful.

Tetracylines. The anti-amyloidogenic activity of anthracycline iododoxorubicin is evidenced by its capability to bind PrP amyloid, disrupt spontaneous PrP-peptide fibrilisation and reduce infectivity of prion-infected brain, and has prompted research into analogs readily used in clinical practice, capable of crossing the BBB and showing potential anti-prion activity (reviewed in [154]). The foundation for investigating tetracycline antibiotics was the structural homology with the algycone moiety of the anthracyclines and the prion interacting compounds Congo red and tetrapyrroles. Tetracyclines have limited toxicity and the derivatives doxycycline and minocycline are capable of efficiently crossing the BBB. *In vitro* tetracycline can interact with amyloid fibrils generated with synthetic peptides homologous to human PrP sequence aa 106-126 and 82-146, which mimic the central features of the proteinase K resisitant core of PrP^{Sc}, resulting in diminished (dependant on derivative of tetracycline used) proteinase K resistance of the peptide amyloid fibrils. Incubation of tetracycline hydrochloride and doxycycline hyclate with partially purified PrP^{Sc} from CJD and vCJD patients as well as from cattle with BSE also resulted in diminished proteinase K resistance. Furthermore tetracylines inhibited neuronal death and astroglial proliferation induced by PrP peptides *in vitro*. To test the effects of tetracyclines *in vivo* 263K scrapie-infected brain homogenates were preincubated with tetracycline or doxycycline before intercerebral inoculation of healthy hamsters, resulting in significant delayed onset of clinical disease and prolonged survival. Furthermore, doxycycline administrations lead to prolonged survival when administered 30 days after prion inoculation (albeit less compared to when doxycycline was pre-incubated). However, these results do support the potential anti-prion activity of tetracyclines and its structural analogs *in vivo*. Preliminary data from observational studies in humans, in which doxycycline is administered for compassionate reasons, show significantly prolonged survival of CJD patients, confirming the anti-prion activity of tetracyclines in humans. Currently, a drug vs. placebo clinical study is underway with results expected by the end of 2010.

Ideally, in order to develop a successful prion disease therapy all the following stipulations should be fulfilled: the therapeutic must 1) be capable of crossing the blood brain barrier (BBB) or can be delivered to the central nervous system (CNS), 2) be effective at physiological concentrations and non-toxic after biological build-up (during administration), 3) be effective after PrP^{Sc} accumulation has occurred (preferably aiding PrP^{Sc} clearance) and 4) leave PrP^{C} functionality (partially) intact or at least allows restoration of PrP^{C} function after treatment. Unfortunately there are thus far no effective *in vivo* demonstrations of the therapeutics described above (acridine and phenothiazin derivatives), are only usable as a prophylactic (passive immunization, heparan mimetics, β-breaker peptides), or do not conform to one or more of the stipulations described for a drug: difficulty in passing BBB, sensitivity to proteolytic degradation (PrP-peptides, β-breaker peptides), and delivery to the CNS (PrP-peptides, β-breaker peptides, shRNA (siRNA), heterelogous PrP). However, some promising prospects still do exist. shRNA (siRNA) may prove useful when it can be delivered throughout the CNS and if the effects can be reversed (PrP^{C} functionality restored). Further research has to be performed to find out whether branched polyamines are viable candidates for application *in vivo*. Preliminary results of patient treatment with tetracyclines are encouraging and results form the drug vs. placebo clinical studies should provide insights in the therapeutic value of the compound. Furthermore, investigation of calpain dependant cleavage may lead to new strategies that allow clearance of already formed PrP^{Sc}, which alone or in combination with another therapeutic may prove capable of curing prion disease. All testing of possible new therapeutic strategies are seriously hampered by the extreme long incubation periods and late diagnosis of natural infections (mostly post-mortum).

Concluding remarks.

At a first glance it seems that PrP^C is a fairly promiscuous protein interacting with a multitude of molecules, including several metal ions as well as many different proteins. Closer scrutiny of the data published to date allows for a more careful conclusion. Firstly, PrP interacts with several proteins that are probably part of the normal PrP biological life cycle. The 'regular' PrP associated proteins include proteins involved in binding/ internalization of PrP (LRP/LR, HSPG [GAGs], clathrin and LRP1) likely modulated by the copper-load of the N-terminal octarepeats of PrP.

A probable physiological function of PrP^C can be extrapolated from the interactions of PrP^C with different proteins that have a known function in signalling pathways involved in cell-survival and/or differentiation. The emerging overall picture of the physiological function of PrP^C seems that PrP^C is part of an intricate and complex macromolecular complex in which PrP^C plays a pivotal role. Depending on the protein that PrP^C interacts with, different pathways are initiated. These pathways can lead to differentiation of a particular cell or lead to protection of a particular cell by preventing apoptosis. When the latter pathway is interrupted, either because PrP^C is not available for interaction or because PrP^C interacts with proteins involved in pathways initiating apoptosis, remains to be elucidated. However, by further investigating interactions of PrP^C with itself or with other proteins, in time, a complete picture of the physiological function of PrP^C should emerge and should open up new insights that allow development of new and different modes of inhibiting prion protein conversion.

To date most of the promising therapeutic strategies are either only useful as a prophylactic or the applicability *in vivo* still needs to be determined. Preliminary results with tetracyclines as a therapeutic compound seem promising for prolonging survival of CJD patients. However, whether tetracyclines can cure human TSE remains to be determined. In hamsters tetracyclines were only capable preventing development of disease when pre-incubated with the inoculum. Efficacies of tetracyclines (or any compound) as a therapeutic is seriously hampered by the long incubation period of TSEs and the (current) inability to timely diagnose TSEs. Therefore pre-clinical diagnosis of TSEs is of the outmost importance, which poses a problem, for diagnosis of TSEs remains difficult; the clinical features are not exclusive for CJD but also applicable to several different (neurological) disorders. Only in the final stages of disease is clinical diagnosis more specific (not definitive), but the only conclusive determination of prion disease is a post-mortem test detecting PrP^{Sc}. Therefore, elucidating the molecular processes involved in conversion should give rise to different modes of intervention.

Focussing on the (stabilizing) self-interaction domains of PrP as well as the molecular processes involved in conversion may lead to further therapeutic strategies which may well allow for retention of the physiological function(s) of PrP^{C}. Hopefully current promising therapeutics or the aforementioned new therapeutic strategies prove to be not only effective prophylactically but also after clinical onset of prion disease. In addition further research should focus on improving diagnosis/detection of prion disease, in order to allow early intervention. Taken together research is focussing on more effective therapeutic strategies capable of blocking of PrP^{Sc} formation and promoting PrP^{Sc} clearance. If therapy is started before onset of major clinical problems the therapeutic therapy may even ultimately result in symptomatic reversal, emphazising that a conclusive preclinical diagnostic tool is indispensable.

References.

1. Griffith JS. *Self-replication and scrapie.* Nature 1967;215(105):1043-4.
2. Prusiner SB. *Novel proteinaceous infectious particles cause scrapie.* Science 1982;216(4542):136-44.
3. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A et al. *A new variant of Creutzfeldt-Jakob disease in the UK.* Lancet 1996;347(9006):921-5.
4. Harris DA. *Trafficking, turnover and membrane topology of PrP.* Br Med Bull 2003;66:71-85.
5. Harris DA. Cellular biology of prion diseases. Clin Microbiol Rev 1999;12(3):429-44.
6. Kaneko K, Vey M, Scott M, Pilkuhn S, Cohen FE, Prusiner SB. *COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform.* Proc Natl Acad Sci U S A 1997;94(6):2333-8.
7. Hegde RS, Tremblay P, Groth D, DeArmond SJ, Prusiner SB, Lingappa VR. *Transmissible and genetic prion diseases share a common pathway of neurodegeneration.* Nature 1999;402(6763):822-6.
8. Gu Y, Luo X, Basu S, Fujioka H, Singh N. *Cell-specific metabolism and pathogenesis of transmembrane prion protein.* Mol Cell Biol 2006;26(7):2697-715.
9. Riesner D. *Biochemistry and structure of PrP(C) and PrP(Sc).* Br Med Bull 2003;66:21-33.
10. van Keulen LJ, Schreuder BE, Vromans ME, Langeveld JP, Smits MA. *Pathogenesis of natural scrapie in sheep.* Arch Virol Suppl 2000(16):57-71.
11. Cashman NR, Caughey B. *Prion diseases--close to effective therapy?* Nat Rev Drug Discov 2004;3(10):874-84.
12. van Keulen LJ, Bossers A, van Zijderveld F. *TSE pathogenesis in cattle and sheep.* Vet Res 2008;39(4):24.
13. van Keulen LJ, Vromans ME, Dolstra CH, Bossers A, van Zijderveld FG. *Pathogenesis of bovine spongiform encephalopathy in sheep.* Arch Virol 2008;153(3):445-53.
14. Horiuchi M, Yamazaki N, Ikeda T, Ishiguro N, Shinagawa M. *A cellular form of prion protein (PrPC) exists in many non-neuronal tissues of sheep.* J Gen Virol 1995;76 (Pt 10):2583-7.
15. Silva JL, Lima LM, Foguel D, Cordeiro Y. *Intriguing nucleic-acid-binding features of mammalian prion protein.* Trends Biochem Sci 2008;33(3):132-40.
16. Rieger R, Edenhofer F, Lasmezas CI, Weiss S. *The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells.* Nat Med 1997;3(12):1383-8.
17. Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, Rieger R et al. *The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein.* Embo J 2001;20(21):5863-75.

18. Gauczynski S, Nikles D, El-Gogo S, Papy-Garcia D, Rey C, Alban S et al. *The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes.* J Infect Dis 2006;194(5):702-9.
19. Hundt C, Peyrin JM, Haik S, Gauczynski S, Leucht C, Rieger R et al. *Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor.* Embo J 2001;20(21):5876-86.
20. Shyng SL, Moulder KL, Lesko A, Harris DA. *The N-terminal domain of a glycolipid-anchored prion protein is essential for its endocytosis via clathrin-coated pits.* J Biol Chem 1995;270(24):14793-800.
21. Sunyach C, Jen A, Deng J, Fitzgerald KT, Frobert Y, Grassi J et al. *The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein.* Embo J 2003;22(14):3591-601.
22. Taylor DR, Watt NT, Perera WS, Hooper NM. *Assigning functions to distinct regions of the N-terminus of the prion protein that are involved in its copper-stimulated, clathrin-dependent endocytosis.* J Cell Sci 2005;118(Pt 21):5141-53.
23. Taylor DR, Hooper NM. *The low-density lipoprotein receptor-related protein 1 (LRP1) mediates the endocytosis of the cellular prion protein.* Biochem J 2007;402(1):17-23.
24. Parkyn CJ, Vermeulen EG, Mootoosamy RC, Sunyach C, Jacobsen C, Oxvig C et al. *LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein.* J Cell Sci 2008;121(Pt 6):773-83.
25. Roucou X, LeBlanc AC. *Cellular prion protein neuroprotective function: implications in prion diseases.* J Mol Med 2005;83(1):3-11.
26. Li A, Harris DA. *Mammalian prion protein suppresses Bax-induced cell death in yeast.* J Biol Chem 2005;280(17):17430-4.
27. Zanata SM, Lopes MH, Mercadante AF, Hajj GN, Chiarini LB, Nomizo R et al. *Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection.* Embo J 2002;21(13):3307-16.
28. Lopes MH, Hajj GN, Muras AG, Mancini GL, Castro RM, Ribeiro KC et al. *Interaction of cellular prion and stress-inducible protein 1 promotes neuritogenesis and neuroprotection by distinct signaling pathways.* J Neurosci 2005;25(49):11330-9.
29. Caetano FA, Lopes MH, Hajj GN, Machado CF, Pinto Arantes C, Magalhaes AC et al. *Endocytosis of prion protein is required for ERK1/2 signaling induced by stress-inducible protein 1.* J Neurosci 2008;28(26):6691-702.
30. Coitinho AS, Lopes MH, Hajj GN, Rossato JI, Freitas AR, Castro CC et al. *Short-term memory formation and long-term memory consolidation are enhanced by cellular prion association to stress-inducible protein 1.* Neurobiol Dis 2007;26(1):282-90.

31. Weise J, Sandau R, Schwarting S, Crome O, Wrede A, Schulz-Schaeffer W et al. *Deletion of cellular prion protein results in reduced Akt activation, enhanced postischemic caspase-3 activation, and exacerbation of ischemic brain injury.* Stroke 2006;37(5):1296-300.

32. Coitinho AS, Roesler R, Martins VR, Brentani RR, Izquierdo I. *Cellular prion protein ablation impairs behavior as a function of age.* Neuroreport 2003;14(10):1375-9.

33. Criado JR, Sanchez-Alavez M, Conti B, Giacchino JL, Wills DN, Henriksen SJ et al. *Mice devoid of prion protein have cognitive deficits that are rescued by reconstitution of PrP in neurons.* Neurobiol Dis 2005;19(1-2):255-65.

34. Mouillet-Richard S, Schneider B, Pradines E, Pietri M, Ermonval M, Grassi J et al. *Cellular prion protein signaling in serotonergic neuronal cells.* Ann N Y Acad Sci 2007;1096:106-19.

35. Mattei V, Garofalo T, Misasi R, Circella A, Manganelli V, Lucania G et al. *Prion protein is a component of the multimolecular signaling complex involved in T cell activation.* FEBS Lett 2004;560(1-3):14-8.

36. Santuccione A, Sytnyk V, Leshchyns'ka I, Schachner M. *Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth.* J Cell Biol 2005;169(2):341-54.

37. Graner E, Mercadante AF, Zanata SM, Martins VR, Jay DG, Brentani RR. *Laminin-induced PC-12 cell differentiation is inhibited following laser inactivation of cellular prion protein.* FEBS Lett 2000;482(3):257-60.

38. Satoh J, Obayashi S, Misawa T, Sumiyoshi K, Oosumi K, Tabunoki H. *Protein microarray analysis identifies human cellular prion protein interactors.* Neuropathol Appl Neurobiol 2008.

39. Haigh CL, Lewis VA, Vella LJ, Masters CL, Hill AF, Lawson VA et al. *PrPC-related signal transduction is influenced by copper, membrane integrity and the alpha cleavage site.* Cell Res 2009;19(9):1062-78.

40. Stohr J, Weinmann N, Wille H, Kaimann T, Nagel-Steger L, Birkmann E et al. *Mechanisms of prion protein assembly into amyloid.* Proc Natl Acad Sci U S A 2008;105(7):2409-14.

41. Caughey B, Kocisko DA, Raymond GJ, T. LP,Jr. *Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state.* Chem Biol 1995;2(12):807-17.

42. Wille H, Michelitsch MD, Guenebaut V, Supattapone S, Serban A, Cohen FE et al. *Structural studies of the scrapie prion protein by electron crystallography.* Proc Natl Acad Sci U S A 2002;99(6):3563-8.

43. Govaerts C, Wille H, Prusiner SB, Cohen FE. *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A 2004;101(22):8342-7.

44. Langedijk JP, Fuentes G, Boshuizen R, Bonvin AM. *Two-rung model of a left-handed beta-helix for prions explains species barrier and strain variation in transmissible spongiform encephalopathies.* J Mol Biol 2006;360(4):907-20.
45. Jenkins DC, Sylvester ID, Pinheiro TJ. *The elusive intermediate on the folding pathway of the prion protein.* Febs J 2008;275(6):1323-35.
46. Hosszu LL, Trevitt CR, Jones S, Batchelor M, Scott DJ, Jackson GS et al. *Conformational properties of beta -PrP.* J Biol Chem 2009.
47. Gerber R, Tahiri-Alaoui A, Hore PJ, James W. *Oligomerization of the human prion protein proceeds via a molten globule intermediate.* J Biol Chem 2007;282(9):6300-7.
48. Apetri AC, Maki K, Roder H, Surewicz WK. *Early intermediate in human prion protein folding as evidenced by ultrarapid mixing experiments.* J Am Chem Soc 2006;128(35):11673-8.
49. Silveira JR, Hughson AG, Caughey B. *Fractionation of prion protein aggregates by asymmetrical flow field-flow fractionation.* Methods Enzymol 2006;412:21-33.
50. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT et al. *Cell-free formation of protease-resistant prion protein.* Nature 1994;370(6489):471-4.
51. Caughey B. *Interactions between prion protein isoforms: the kiss of death?* Trends Biochem Sci 2001;26(4):235-42.
52. Raymond GJ, Bossers A, Raymond LD, O'Rourke KI, McHolland LE, Bryant PK,3rd et al. *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo J 2000;19(17):4425-30.
53. Supattapone S. *Prion protein conversion in vitro.* J Mol Med 2004;82(6):348-56.
54. Castilla J, Morales R, Saa P, Barria M, Gambetti P, Soto C. *Cell-free propagation of prion strains.* Embo J 2008.
55. Weber P, Giese A, Piening N, Mitteregger G, Thomzig A, Beekes M et al. *Generation of genuine prion infectivity by serial PMCA.* Vet Microbiol 2007;123(4):346-57.
56. Deleault NR, Harris BT, Rees JR, Supattapone S. *Formation of native prions from minimal components in vitro.* Proc Natl Acad Sci U S A 2007;104(23):9741-6.
57. Baskakov IV, Legname G, Baldwin MA, Prusiner SB, Cohen FE. *Pathway complexity of prion protein assembly into amyloid.* J Biol Chem 2002;277(24):21140-8.
58. Lu BY, Chang JY. *Isolation of isoforms of mouse prion protein with PrP(SC)-like structural properties.* Biochemistry 2001;40(44):13390-6.
59. Bossers A, de Vries R, Smits MA. *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol 2000;74(3):1407-14.
60. Dubois MA, Sabatier P, Durand B, Calavas D, Ducrot C, Chalvet-Monfray K. *Multiplicative genetic effects in scrapie disease susceptibility.* C R Biol 2002;325(5):565-70.

61. Sabuncu E, Petit S, Le Dur A, Lan Lai T, Vilotte JL, Laude H et al. *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol 2003;77(4):2696-700.

62. Tranulis MA. *Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep.* Apmis 2002;110(1):33-43.

63. Rigter A, Bossers A. *Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion.* J Gen Virol 2005;86(Pt 9):2627-34.

64. Rigter A, Langeveld JP, Timmers-Parohi D, Jacobs JG, Moonen PL, Bossers A. *Mapping of possible prion protein self-interaction domains using peptide arrays.* BMC Biochem 2007;8:6.

65. Rigter A, Priem J, Timmers-Parohi D, Langeveld JP, van Zijderveld FG, Bossers A. *Prion protein self-peptides modulate prion interactions and conversion.* BMC Biochem 2009;10:29.

66. Jacobs JG, Langeveld JP, Biacabe AG, Acutis PL, Polak MP, Gavier-Widen D et al. *Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe.* J Clin Microbiol 2007;45(6):1821-9.

67. Thuring CM, Erkens JH, Jacobs JG, Bossers A, Van Keulen LJ, Garssen GJ et al. *Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein.* J Clin Microbiol 2004;42(3):972-80.

68. Moroncini G, Kanu N, Solforosi L, Abalos G, Telling GC, Head M et al. *Motif-grafted antibodies containing the replicative interface of cellular PrP are specific for PrPSc.* Proc Natl Acad Sci U S A 2004;101(28):10404-9.

69. Riek R, Hornemann S, Wider G, Glockshuber R, Wuthrich K. *NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231).* 1997;282; 413(2):8.

70. Solforosi L, Bellon A, Schaller M, Cruite JT, Abalos GC, Williamson RA. *Toward Molecular Dissection of PrPC-PrPSc Interactions.* J Biol Chem 2007;282(10):7465-71.

71. Surewicz WK, Jones EM, Apetri AC. *The emerging principles of Mammalian prion propagation and transmissibility barriers: insight from studies in vitro.* Acc Chem Res 2006;39(9):654-62.

72. Beringue V, Vilette D, Mallinson G, Archer F, Kaisar M, Tayebi M et al. *PrPSc binding antibodies are potent inhibitors of prion replication in cell lines.* J Biol Chem 2004;279(38):39671-6.

73. Enari M, Flechsig E, Weissmann C. *Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody.* Proc Natl Acad Sci U S A 2001;98(16):9295-9.

74. Korth C, Stierli B, Streit P, Moser M, Schaller O, Fischer R et al. *Prion (PrPSc)-specific epitope defined by a monoclonal antibody.* Nature 1997;390(6655):74-7.

75. Peretz D, Williamson RA, Kaneko K, Vergara J, Leclerc E, Schmitt-Ulms G et al. *Antibodies inhibit prion propagation and clear cell cultures of prion infectivity.* Nature 2001;412(6848):739-43.

76. Perrier V, Solassol J, Crozet C, Frobert Y, Mourton-Gilles C, Grassi J et al. *Anti-PrP antibodies block PrPSc replication in prion-infected cell cultures by accelerating PrPC degradation.* J Neurochem 2004;89(2):454-63.

77. Heppner FL, Musahl C, Arrighi I, Klein MA, Rulicke T, Oesch B et al. *Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies.* Science 2001;294(5540):178-82.

78. Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M et al. *Mice devoid of PrP are resistant to scrapie.* Cell 1993;73(7):1339-47.

79. Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S, Collinge J. *Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis.* Science 2003;302(5646):871-4.

80. Bossers A, Belt P, Raymond GJ, Caughey B, de Vries R, Smits MA. *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A 1997;94(10):4931-6.

81. Daude N, Marella M, Chabry J. *Specific inhibition of pathological prion protein accumulation by small interfering RNAs.* J Cell Sci 2003;116(Pt 13):2775-9.

82. Pfeifer A, Eigenbrod S, Al-Khadra S, Hofmann A, Mitteregger G, Moser M et al. *Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice.* J Clin Invest 2006;116(12):3204-10.

83. White MD, Farmer M, Mirabile I, Brandner S, Collinge J, Mallucci GR. *Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease.* Proc Natl Acad Sci U S A 2008;105(29):10238-43.

84. Singer O, Yanai A, Verma IM. *Silence of the genes.* Proc Natl Acad Sci U S A 2004;101(15):5313-4.

85. Wiznerowicz M, Trono D. *Harnessing HIV for therapy, basic research and biotechnology.* Trends Biotechnol 2005;23(1):42-7.

86. Horiuchi M, Priola SA, Chabry J, Caughey B. *Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers.* Proc Natl Acad Sci U S A 2000;97(11):5836-41.

87. Palmer MS, Dryden AJ, Hughes JT, Collinge J. *Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease.* Nature 1991;352(6333):340-2.

88. Bossers A. *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie.* 1999.

89. Holscher C, Delius H, Burkle A. *Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation.* J Virol 1998;72(2):1153-9.

90. Caplazi PA, O'Rourke KI, Baszler TV. *Resistance to scrapie in PrP ARR/ARQ heterozygous sheep is not caused by preferential allelic use.* J Clin Pathol 2004;57(6):647-50.

91. Groschup MH, Lacroux C, Buschmann A, Luhken G, Mathey J, Eiden M et al. *Classic scrapie in sheep with the ARR/ARR prion genotype in Germany and France.* Emerg Infect Dis 2007;13(8):1201-7.

92. Le Dur A, Beringue V, Andreoletti O, Reine F, Lai TL, Baron T et al. *A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes.* Proc Natl Acad Sci U S A 2005;102(44):16031-6.

93. Buschmann A, Luhken G, Schultz J, Erhardt G, Groschup MH. *Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR).* J Gen Virol 2004;85(Pt 9):2727-33.

94. Baylis M, McIntyre KM. *Transmissible spongiform encephalopathies: scrapie control under new strain.* Nature 2004;432(7019):810-1.

95. Toupet K, Compan V, Crozet C, Mourton-Gilles C, Mestre-Frances N, Ibos F et al. *Effective gene therapy in a mouse model of prion diseases.* PLoS ONE 2008;3(7):e2773.

96. Chabry J, Priola SA, Wehrly K, Nishio J, Hope J, Chesebro B. *Species-independent inhibition of abnormal prion protein (PrP) formation by a peptide containing a conserved PrP sequence.* J Virol 1999;73(8):6245-50.

97. Mohan J, Bruce ME, Mabbott NA. *Follicular dendritic cell dedifferentiation reduces scrapie susceptibility following inoculation via the skin.* Immunology 2005;114(2):225-34.

98. Glaysher BR, Mabbott NA. *Role of the GALT in scrapie agent neuroinvasion from the intestine.* J Immunol 2007;178(6):3757-66.

99. van Keulen LJ, Vromans ME, van Zijderveld FG. *Early and late pathogenesis of natural scrapie infection in sheep.* Apmis 2002;110(1):23-32.

100. Heppner FL, Christ AD, Klein MA, Prinz M, Fried M, Kraehenbuhl JP et al. *Transepithelial prion transport by M cells.* Nat Med 2001;7(9):976-7.

101. Mishra RS, Basu S, Gu Y, Luo X, Zou WQ, Mishra R et al. *Protease-resistant human prion protein and ferritin are cotransported across Caco-2 epithelial cells: implications for species barrier in prion uptake from the intestine.* J Neurosci 2004;24(50):11280-90.

102. Raymond CR, Aucouturier P, Mabbott NA. *In vivo depletion of CD11c+ cells impairs scrapie agent neuroinvasion from the intestine.* J Immunol 2007;179(11):7758-66.

103. Mabbott NA, Young J, McConnell I, Bruce ME. *Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility.* J Virol 2003;77(12):6845-54.

104. Montrasio F, Frigg R, Glatzel M, Klein MA, Mackay F, Aguzzi A et al. *Impaired prion replication in spleens of mice lacking functional follicular dendritic cells.* Science 2000;288(5469):1257-9.

105. Supattapone S, Wille H, Uyechi L, Safar J, Tremblay P, Szoka FC et al. *Branched polyamines cure prion-infected neuroblastoma cells.* J Virol 2001;75(7):3453-61.

106. Solassol J, Crozet C, Perrier V, Leclaire J, Beranger F, Caminade AM et al. *Cationic phosphorus-containing dendrimers reduce prion replication both in cell culture and in mice infected with scrapie.* J Gen Virol 2004;85(Pt 6):1791-9.

107. Oishi T, Hagiwara K, Kinumi T, Yamakawa Y, Nishijima M, Nakamura K et al. *Effects of beta-sheet breaker peptide polymers on scrapie-infected mouse neuroblastoma cells and their affinities to prion protein fragment PrP(81-145).* Org Biomol Chem 2003;1(15):2626-9.

108. Reilly CE. *Beta-sheet breaker peptides reverse conformation of pathogenic prion proteins.* J Neurol 2000;247(4):319-20.

109. Soto C, Kascsak RJ, Saborio GP, Aucouturier P, Wisniewski T, Prelli F et al. *Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides.* Lancet 2000;355(9199):192-7.

110. Harris DA, Huber MT, van Dijken P, Shyng SL, Chait BT, Wang R. *Processing of a cellular prion protein: identification of N- and C-terminal cleavage sites.* Biochemistry 1993;32(4):1009-16.

111. Pan KM, Stahl N, Prusiner SB. *Purification and properties of the cellular prion protein from Syrian hamster brain.* Protein Sci 1992;1(10):1343-52.

112. Taraboulos A, Scott M, Semenov A, Avrahami D, Laszlo L, Prusiner SB. *Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform.* J Cell Biol 1995;129(1):121-32.

113. Yadavalli R, Guttmann RP, Seward T, Centers AP, Williamson RA, Telling GC. *Calpain-dependent endoproteolytic cleavage of PrPSc modulates scrapie prion propagation.* J Biol Chem 2004;279(21):21948-56.

114. Wang X, Wang F, Sy MS, Ma J. *Calpain and other cytosolic proteases can contribute to the degradation of retro-translocated prion protein in the cytosol.* J Biol Chem 2005;280(1):317-25.

115. Kristensson K, Feuerstein B, Taraboulos A, Hyun WC, Prusiner SB, DeArmond SJ. *Scrapie prions alter receptor-mediated calcium responses in cultured cells.* Neurology 1993;43(11):2335-41.

116. O'Donovan CN, Tobin D, Cotter TG. *Prion protein fragment PrP-(106-126) induces apoptosis via mitochondrial disruption in human neuronal SH-SY5Y cells.* J Biol Chem 2001;276(47):43516-23.

117. Gabizon R, McKinley MP, Groth D, Prusiner SB. *Immunoaffinity purification and neutralization of scrapie prion infectivity.* Proc Natl Acad Sci U S A 1988;85(18):6617-21.

118. Horiuchi M, Caughey B. *Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state.* Embo J 1999;18(12):3193-203.

119. Sigurdsson EM, Brown DR, Daniels M, Kascsak RJ, Kascsak R, Carp R et al. *Immunization delays the onset of prion disease in mice.* Am J Pathol 2002;161(1):13-7.

120. Sigurdsson EM, Sy MS, Li R, Scholtzova H, Kascsak RJ, Kascsak R et al. *Anti-prion antibodies for prophylaxis following prion exposure in mice.* Neurosci Lett 2003;336(3):185-7.

121. Schwarz A, Kratke O, Burwinkel M, Riemer C, Schultz J, Henklein P et al. *Immunisation with a synthetic prion protein-derived peptide prolongs survival times of mice orally exposed to the scrapie agent.* Neurosci Lett 2003;350(3):187-9.

122. Nikles D, Bach P, Boller K, Merten CA, Montrasio F, Heppner FL et al. *Circumventing tolerance to the prion protein (PrP): vaccination with PrP-displaying retrovirus particles induces humoral immune responses against the native form of cellular PrP.* J Virol 2005;79(7):4033-42.

123. Handisurya A, Gilch S, Winter D, Shafti-Keramat S, Maurer D, Schatzl HM et al. *Vaccination with prion peptide-displaying papillomavirus-like particles induces autoantibodies to normal prion protein that interfere with pathologic prion protein production in infected cells.* Febs J 2007;274(7):1747-58.

124. White AR, Enever P, Tayebi M, Mushens R, Linehan J, Brandner S et al. *Monoclonal antibodies inhibit prion replication and delay the development of prion disease.* Nature 2003;422(6927):80-3.

125. Cardinale A, Biocca S. *The potential of intracellular antibodies for therapeutic targeting of protein-misfolding diseases.* Trends Mol Med 2008;14(9):373-80.

126. Solforosi L, Criado JR, McGavern DB, Wirz S, Sanchez-Alavez M, Sugama S et al. *Cross-linking cellular prion protein triggers neuronal apoptosis in vivo.* Science 2004;303(5663):1514-6.

127. Farquhar CF, Dickinson AG. *Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection.* J Gen Virol 1986;67 (Pt 3):463-73.

128. Caughey B, Raymond GJ. *Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells.* J Virol 1993;67(2):643-50.

129. Adjou KT, Simoneau S, Sales N, Lamoury F, Dormont D, Papy-Garcia D et al. *A novel generation of heparan sulfate mimetics for the treatment of prion diseases.* J Gen Virol 2003;84(Pt 9):2595-603.

130. Larramendy-Gozalo C, Barret A, Daudigeos E, Mathieu E, Antonangeli L, Riffet C et al. *Comparison of CR36, a new heparan mimetic, and pentosan polysulfate in the treatment of prion diseases.* J Gen Virol 2007;88(Pt 3):1062-7.

131. Todd NV, Morrow J, Doh-ura K, Dealler S, O'Hare S, Farling P et al. *Cerebroventricular infusion of pentosan polysulphate in human variant Creutzfeldt-Jakob disease.* J Infect 2005;50(5):394-6.

132. Whittle IR, Knight RS, Will RG. *Unsuccessful intraventricular pentosan polysulphate treatment of variant Creutzfeldt-Jakob disease.* Acta Neurochir (Wien) 2006;148(6):677,9; discussion 679.

133. Bone I, Belton L, Walker AS, Darbyshire J. *Intraventricular pentosan polysulphate in human prion diseases: an observational study in the UK.* Eur J Neurol 2008;15(5):458-64.

134. Pocchiari M, Ladogana A, Graziano S, Puopolo M. *Creutzfeldt-Jakob disease: hopes for therapy.* Eur J Neurol 2008;15(5):435-6.

135. Poli G, Martino PA, Villa S, Carcassola G, Giannino ML, Dall'Ara P et al. *Evaluation of anti-prion activity of congo red and its derivatives in experimentally infected hamsters.* Arzneimittelforschung 2004;54(7):406-15.

136. Caughey B, Race RE. *Potent inhibition of scrapie-associated PrP accumulation by congo red.* J Neurochem 1992;59(2):768-71.

137. Sellarajah S, Lekishvili T, Bowring C, Thompsett AR, Rudyk H, Birkett CR et al. *Synthesis of analogues of Congo red and evaluation of their anti-prion activity.* J Med Chem 2004;47(22):5515-34.

138. Caughey B, Raymond LD, Raymond GJ, Maxson L, Silveira J, Baron GS. *Inhibition of protease-resistant prion protein accumulation in vitro by curcumin.* J Virol 2003;77(9):5499-502.

139. Riemer C, Burwinkel M, Schwarz A, Gultner S, Mok SW, Heise I et al. *Evaluation of drugs for treatment of prion infections of the central nervous system.* J Gen Virol 2008;89(Pt 2):594-7.

140. Caughey WS, Raymond LD, Horiuchi M, Caughey B. *Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines.* Proc Natl Acad Sci U S A 1998;95(21):12117-22.

141. Priola SA, Raines A, Caughey WS. *Porphyrin and phthalocyanine antiscrapie compounds.* Science 2000;287(5457):1503-6.

142. Priola SA, Raines A, Caughey W. *Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice.* J Infect Dis 2003;188(5):699-705.

143. Barret A, Tagliavini F, Forloni G, Bate C, Salmona M, Colombo L et al. *Evaluation of quinacrine treatment for prion diseases.* J Virol 2003;77(15):8462-9.

144. Doh-Ura K, Iwaki T, Caughey B. *Lysosomotropic agents and cysteine protease inhibitors inhibit scrapie-associated prion protein accumulation.* J Virol 2000;74(10):4894-7.

145. Doh-ura K, Ishikawa K, Murakami-Kubo I, Sasaki K, Mohri S, Race R et al. *Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models.* J Virol 2004;78(10):4999-5006.

146. Korth C, May BC, Cohen FE, Prusiner SB. *Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease.* Proc Natl Acad Sci U S A 2001;98(17):9836-41.

147. Murakami-Kubo I, Doh-Ura K, Ishikawa K, Kawatake S, Sasaki K, Kira J et al. *Quinoline derivatives are therapeutic candidates for transmissible spongiform encephalopathies.* J Virol 2004;78(3):1281-8.

148. Kobayashi Y, Hirata K, Tanaka H, Yamada T. *[Quinacrine administration to a patient with Creutzfeldt-Jakob disease who received a cadaveric dura mater graft--an EEG evaluation].* Rinsho Shinkeigaku 2003;43(7):403-8.

149. Nakajima M, Yamada T, Kusuhara T, Furukawa H, Takahashi M, Yamauchi A et al. *Results of quinacrine administration to patients with Creutzfeldt-Jakob disease.* Dement Geriatr Cogn Disord 2004;17(3):158-63.

150. Martinez-Lage JF, Rabano A, Bermejo J, Martinez Perez M, Guerrero MC, Contreras MA et al. *Creutzfeldt-Jakob disease acquired via a dural graft: failure of therapy with quinacrine and chlorpromazine.* Surg Neurol 2005;64(6):542,5, discussion 545.

151. Wroe SJ, Pal S, Siddique D, Hyare H, Macfarlane R, Joiner S et al. *Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report.* Lancet 2006;368(9552):2061-7.

152] Satoh K, Shirabe S, Eguchi K, Yamauchi A, Kataoka Y, Niwa M et al. *Toxicity of quinacrine can be reduced by co-administration of P-glycoprotein inhibitor in sporadic Creutzfeldt-Jakob disease.* Cell Mol Neurobiol 2004;24(6):873-5.

153. Scoazec JY, Krolak-Salmon P, Casez O, Besson G, Thobois S, Kopp N et al. *Quinacrine-induced cytolytic hepatitis in sporadic Creutzfeldt-Jakob disease.* Ann Neurol 2003;53(4):546-7.

154. Forloni G, Salmona M, Marcon G, Tagliavini F. *Tetracyclines and prion infectivity.* Infect Disord Drug Targets 2009;9(1):23-30.

155. Morel E, Andrieu T, Casagrande F, Gauczynski S, Weiss S, Grassi J et al. *Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor.* Am J Pathol 2005;167(4):1033-42.

156. Horonchik L, Tzaban S, Ben-Zaken O, Yedidia Y, Rouvinski A, Papy-Garcia D et al. *Heparan sulfate is a cellular receptor for purified infectious prions.* J Biol Chem 2005;280(17):17062-7.

157. Shyng SL, Heuser JE, Harris DA. *A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits.* J Cell Biol 1994;125(6):1239-50.

158. Mouillet-Richard S, Ermonval M, Chebassier C, Laplanche JL, Lehmann S, Launay JM et al. *Signal transduction through prion protein.* Science 2000;289(5486):1925-8.

159. Mouillet-Richard S, Pietri M, Schneider B, Vidal C, Mutel V, Launay JM et al. *Modulation of serotonergic receptor signaling and cross-talk by prion protein.* J Biol Chem 2005;280(6):4592-601.

160. Mattei V, Garofalo T, Misasi R, Gizzi C, Mascellino MT, Dolo V et al. *Association of cellular prion protein with gangliosides in plasma membrane microdomains of neural and lymphocytic cells.* Neurochem Res 2002;27(7-8):743-9.

161. Coitinho AS, Freitas AR, Lopes MH, Hajj GN, Roesler R, Walz R et al. *The interaction between prion protein and laminin modulates memory consolidation.* Eur J Neurosci 2006;24(11):3255-64.

162. Sakudo A, Lee DC, Li S, Nakamura T, Matsumoto Y, Saeki K et al. *PrP cooperates with STI1 to regulate SOD activity in PrP-deficient neuronal cell line.* Biochem Biophys Res Commun 2005;328(1):14-9.

163. Tilly G, Chapuis J, Vilette D, Laude H, Vilotte JL. *Efficient and specific down-regulation of prion protein expression by RNAi.* Biochem Biophys Res Commun 2003;305(3):548-51.

164. Priola SA, Caughey B, Race RE, Chesebro B. *Heterologous PrP molecules interfere with accumulation of protease-resistant PrP in scrapie-infected murine neuroblastoma cells.* J Virol 1994;68(8):4873-8.

165. Chabry J, Caughey B, Chesebro B. *Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides.* J Biol Chem 1998;273(21):13203-7.

166. Mabbott NA, Mackay F, Minns F, Bruce ME. *Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie.* Nat Med 2000;6(7):719-20.

167. Ehlers B, Diringer H. *Dextran sulphate 500 delays and prevents mouse scrapie by impairment of agent replication in spleen.* J Gen Virol 1984;65 (Pt 8):1325-30.

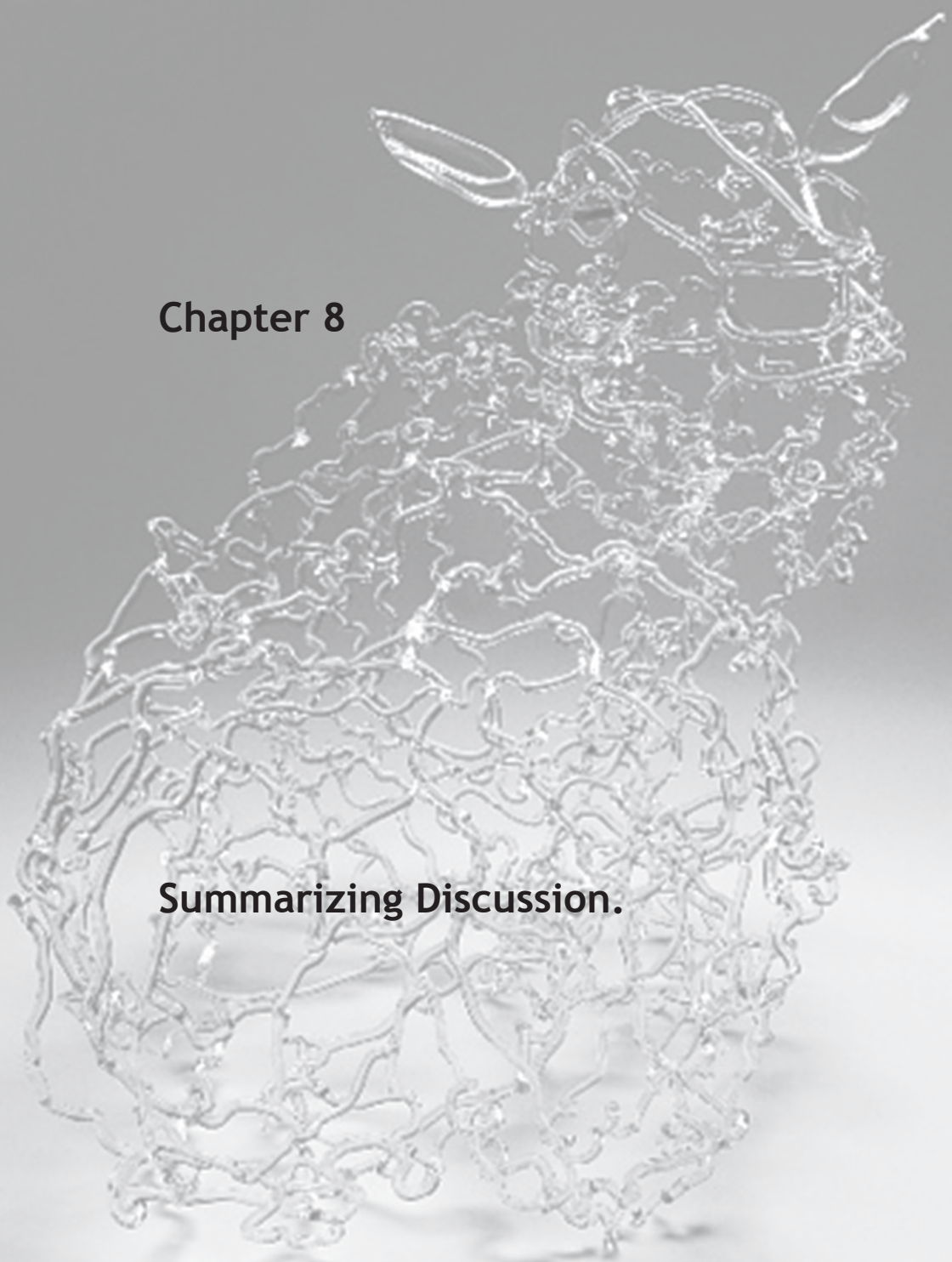
168. Kimberlin RH, Walker CA. *Suppression of scrapie infection in mice by heteropolyanion 23, dextran sulfate, and some other polyanions.* Antimicrob Agents Chemother 1986;30(3):409-13.

169. Gilch S, Winklhofer KF, Groschup MH, Nunziante M, Lucassen R, Spielhaupter C et al. *Intracellular re-routing of prion protein prevents propagation of PrP(Sc) and delays onset of prion disease.* Embo J 2001;20(15):3957-66.

170. Nunziante M, Kehler C, Maas E, Kassack MU, Groschup M, Schatzl HM. *Charged bipolar suramin derivatives induce aggregation of the prion protein at the cell surface and inhibit PrPSc replication.* J Cell Sci 2005;118(Pt 21):4959-73.

171. Caughey WS, Priola SA, Kocisko DA, Raymond LD, Ward A, Caughey B. *Cyclic tetrapyrrole sulfonation, metals, and oligomerization in antiprion activity.* Antimicrob Agents Chemother 2007;51(11):3887-94.

172. Collins SJ, Lewis V, Brazier M, Hill AF, Fletcher A, Masters CL. *Quinacrine does not prolong survival in a murine Creutzfeldt-Jakob disease model.* Ann Neurol 2002;52(4):503-6.



Chapter 8

Summarizing Discussion.

Transmissible spongiform encephalopathy's [TSE] or Prion diseases can occur as a sporadic (sporadic Creutzfeldt Jacob disease [CJD]), hereditary (Gerstmann-Sträussler-Scheinker syndrome, Fatal Familial Insomnia, familial CJD) or infectious (Bovine Spongiform Encephalopathy [BSE], scrapie, iatrogenic CJD, variant CJD) disease in humans and animals. Even though the mechanisms by which these disorders cause disease are not well defined, the common factor between these disorders is the accumulation of disease associated prion protein (PrP^{Sc}) in (mainly) neuronal tissues. The first recorded descriptions of scrapie in sheep were by French and English veterinarians in 1732 and the disease was proven to be infectious in 1936. This makes natural scrapie in sheep the longest studied and probably best candidate to serve as a model for TSEs in general. The first to describe the possibility of an infectious protein as the causative agent in scrapie was Griffith [1], however the term prion (a derivative of proteinacious infectious particle) was introduced by Prusiner [2], whom received a Nobel prize for his protein only theory in 1996. Since the 1988 outbreak of BSE in the United Kingdom research into prion disorders has increased significantly, especially since the emergence of a new variant of Creutzfeldt-Jakob disease (vCJD) which is believed the result of the consumption of BSE infected beef or beef products.

At the beginning of this project the exact molecular mechanism underlying PrP conversion was still unclear as was the means by which polymorphisms in PrP influenced susceptibility and transmissibility. Furthermore, no effective therapeutic for TSEs is available and the emergence of vCJD made TSEs a potential major health risk. This thesis therefore started with the intention to provide a "proof of principle" for the use of conversion-resistant mutant isoforms of the prion protein, in order to interfere in prion replication. The premise, on which use of heterelogous PrP^{C} (in order to interfere with prion replication) is based, is the principle that scrapie susceptibility and transmissibility is largely 'encrypted' in the amino acid sequence of PrP^{C} and PrP^{Sc} itself [3]. To this end Bossers *et al.* had previously developed cell-free *in vitro* conversion assay to asses the effect of scrapie susceptibility-linked polymorphisms on conversion [4, 5].

This cell-free *in vitro* conversion technique was employed to asses several different aspects of prion disease as reviewed in Chapter 2. This chapter offers an overview of the concept of refolding of host-encoded cellular prion protein (PrP^{C}) into the disease associated isoform of the prion protein (PrP^{Sc}), also known as conversion. In particular different *in vitro* techniques (available at the time) for studying conversion are reviewed (cell-culture prion propagation and cell-free prion protein conversion), including the pros and cons of the different techniques. Cell-culture prion propagation studies have provided insights in the kinetics of PrP^{Sc} formation and where PrP conversion occurs.

This technique also proved an excellent system for studying inhibition of conversion and allowed identification of therapeutic compounds. Studies using the cell-free conversion system have provided insights into the underlying molecular mechanisms of conversion, TSE strain variation and inter- and intra-species transmissibility of prion diseases. In particular the effect of polymorphisms on the conversion process is discussed, including some preliminary data of a study that culminated into the paper of chapter 3. Finally, the prospects of therapeutics identified by inhibition of *in vitro* prion protein conversion are discussed.

The first practical study in the realization of this thesis is presented in chapter 3 and aimed to elucidate how disease associated polymorphisms modulate conversion, specifically focusing on understanding the molecular interactions between PrP^{C} and PrP^{Sc} and the effects hereof on subsequent conformational changes that occur. Other studies have shown that the conversion of PrP^{C} by PrP^{Sc} is induced by the aggregated forms of PrP^{Sc} [6, 7] and that the aggregates can be pelleted by centrifugation. Using conditions similar to those used in the cell-free *in vitro* conversion assays, this study used sheep scrapie-susceptibility linked polymorphisms (encoded at amino acid positions 136, 154 and 171) to determine whether differential binding efficiencies of sheep PrP^{C} to PrP^{Sc} are responsible for the observed differential conversion efficiencies of sheep PrP [4, 5, 8, 9]. To this end ^{14}C-labelled PrP^{C} was mixed with purified PrP^{Sc} and subsequently separated into the pellet and supernatant fractions, in order to determine if different polymorphisms resulted in differential binding of PrP^{C} to PrP^{Sc}. The principle hinges on the notion that PrP^{C} binds to PrP^{Sc} (possibly with varying affinity) and can be pelleted together with the PrP^{Sc} aggregates; any PrP^{C} found in pellet fraction must have bound to PrP^{Sc} whereas PrP^{C} remains in the supernatant when unbound and therefore soluble. Several PrP^{Sc} isolates from scrapie infected sheep were isolated from the infected brain. These animals were either homozygous for valine (V) at aa136, alanine (A) at aa154 and a glutamine (Q) at aa171 of PrP^{C} (VRQ) or homozygous for the phylogenetic wild type with alanine at aa136, arginine (R) at aa154 and Q at aa171. Binding with PrP^{Sc} was tested for three natural allelic variants of sheep PrP^{C}; 136V-PrP^{C} (VRQ) associated with increased susceptibility to scrapie, wt-PrP^{C} (ARQ) and 171R-PrP^{C} (ARR), associated with increased resistance to natural scrapie. No significant differences in binding efficiencies were measured between any of the variants, therefore the initial binding efficiencies cannot account for the observed differential conversion efficiencies of sheep scrapie susceptibility linked variants of PrP. For instance, 171R-PrP^{C} binds to either PrP^{Sc} as efficient as 136V-PrP^{C} or wt-PrP^{C}, whereas the conversion efficiency differs markedly (about a quarter to a third of the efficiency of the most efficient conversion reaction). Therefore, not the initial binding step, but a second step (or more) make up

the conversion process involved in conversion of the PrP protein, in which the disease associated polymorphisms have their modulating effect. Since 171R-PrP^C seems to bind as efficient to PrP^{Sc} as wild-type and 136V-PrP^C, the 171R-PrP^C variant may be a valuable tool in providing clues for designing new therapeutic strategies by determination of the mechanism underlying the refolding process of PrP^C into PrP^{Sc} and it may provide a future tool to block prion protein conversion through direct interference of PrP^{Sc} polymer growth.

Whilst studying the polymorphisms effect in conversion we also strived to optimize the cell-free *in vitro* conversion assay in order to measure possible differences in conversion efficiency when conversion was carried out in the presence of heterelogous PrP^C. To achieve this, the absolute amount of converted PrP needed to be increased, with relative differences in conversion efficiency between different isoforms of PrP^C conserved. Unfortunately we discovered that the (at the time only) available cell-free *in vitro* conversion assay was not robust enough increase conversion yields sufficiently to confidently measure differences in conversion efficiency when conversion is carried out in the presence of heterelogous (conversion resistant) PrP^C. This impeded the initial objective to provide a "proof of principle" for the use of conversion-resistant mutant isoforms of the prion protein, in order to interfere in prion replication. However, the exact molecular mechanisms underlying conversion of PrP^C to PrP^{Sc} were still largely unknown. Therefore a more fundamental approach was adopted to elucidate which amino acids of PrP^C were involved in interactions between PrP^C molecules, interactions between PrP^C and PrP^{Sc} and/or (possibly) interactions between PrP^C and other proteins. From literature it became more and more apparent that self-interaction of PrP^C molecules may be at the basis of conversion and therefore prion disease. Consequently the focus was changed to self-interaction domains and the possible effects hereof on the conversion processes, as described in chapters 4 (5) and 6.

Several other studies have shown the importance of the PrP regions encompassing aa90-120 (which includes the amyloidogenic motif AGAAAAGA necessary in conversion of PrP^C into PrP^{Sc} [10]) [11-13] and aa132-156 [12, 14-23]). Furthermore, selective self-interaction between PrP molecules is the most probable basis for initiation of the processes involved in conversion which are potentially influenced by chaperone molecules. Nevertheless the exact mechanisms behind these processes are far from understood. It seems likely that these processes are determined by similarities as well as strain dependent variations in the PrP structure. To our knowledge no attempts had been made to map all possible amino acids (aa) involved in PrP (self-) interaction. The aim of chapter 4 was to systematically map all possible aa involved in PrP self-

interaction (which domains within PrP are capable of interaction). Ovine -and bovine PrP-based solid-phase peptide-arrays consisting of 15-mer overlapping peptides were probed with recombinant sheep PrP^{C}. This study resulted in ten possible consensus binding domains in total, including one domain likely a result of a cross-reaction with the octarepeat domain consensus. For another two domains it remains unclear what their importance is (because of low binding affinity), which left seven domains that are most likely involved in PrP^{C} self-interaction. PrP^{C} was also capable to specifically bind to full length recombinant PrP^{C} as well as brain derived PrP^{C} and PrP^{Sc} in Western blot, confirming the PrP-PrP specific self-interaction observed in the PrP peptide-arrays. Together these results indicate that in addition to direct PrP^{C}-PrP^{Sc} interactions several other molecular interactions between PrP^{C} molecules/sequences themselves may also be possible, facilitating initial steps in the oligomerization process. The PrP peptide-array may additionally facilitate in gaining insight into effects of disease associated polymorphisms in PrP on PrP-PrP binding, and the subsequent molecular conversion of PrP^{C} into PrP^{Sc}. Additionally the technique may provide a tool to investigate the specific effects of compounds (aimed at disrupting PrP-PrP interaction) on PrP^{C} interaction. The (self-) interaction domains described in this chapter may ultimately prove useful in the design of therapeutics interfering with the PrP-PrP binding processes.

The use of the peptide-array assay in this study is novel and prompted a request to submit a chapter for a special edition on "*Peptide Microarrays*" of the journal "*Methods in Molecular Biology*". Therefore a more methodological description of the technique has been supplied in Chapter 5. The chapter entails specific practical details of interest when a peptide-array is used in order to systematically map amino acids involved in interaction of any protein. Because interaction between proteins is central to many biological processes, this novel use of the peptide-array can be applied to investigate binding of many other proteins. Interaction between proteins encompasses interaction with other as well as "self" proteins, resulting in a multitude of effects. Several different 'types and/or results' of interaction can occur; in signal transduction extracellular signals are conferred to the inside of a cell by protein–protein interactions of the signaling molecules. Some proteins form a protein complex with its target protein of interest and this long-term interaction aids transport of the (target) protein between cellular compartments (i.e. importins). Also brief interactions between proteins occur, resulting in modification of the target protein (i.e. post-translational modification), which in itself can change protein–protein interactions. Therefore, interactions between proteins are of importance for most if not all biological processes in living cells. Studying these interactions and elucidating the

contributing amino acid sequences involved in these interactions may lead to improved understanding of the processes underlying disease and may provide the basis for new therapeutic approaches.

Even though we were able to determine domains within PrP^{C} involved in PrP interaction, the molecular mechanisms involved in prion agent replication, converting host-encoded cellular prion protein (PrP^{C}) into the scrapie associated isoform (PrP^{Sc}) remained enigmatic. However, polymorphisms in both PrP isoforms were shown to be of importance in both interspecies and intraspecies transmissibilities [24]. As shown in chapter 4, selective self-interaction between PrP molecules is a probable basis from which the observed differences of PrP^{C} into PrP^{Sc} conversion process (agent replication) can be explained. Formation of PrP^{Sc} aggregates probably requires self-interactions of PrP^{C} molecules as well as with PrP^{Sc} [25, 26]. Thus binding and conformational changes are essential events in this conversion process. Because the study described in Chapter 4 resulted in several interaction domains potentially involved in PrP self-interaction, we aimed to gain further insight in the importance of these domains in conversion. Based on these interaction domains and properties reported in literature, six ovine PrP regions were selected. Chapter 6 describes the assessment of the effects of these peptides on two processes of importance in prion disease development: self-interaction and conversion. To this end effects of the selected peptides were studied in both the peptide-array and the protein misfolding cyclic amplification (PMCA) assay. This study corroborate suggestions that the region containing the polymorphisms at aa position 136, 154 and 171 as well as the peptide-mapping interaction domain [140-PLIHFGNDY-148] (ovine numbering used throughout, chapter 4) is most relevant in differential TSE susceptibility and that stability of this PrP region is a crucial determinant in PrP^{C} conversion efficiency (affecting species-barrier and/or scrapie susceptibility). This study further suggests that the octarepeats, the glycosaminoglycan binding -and the amyloidogenic motif indirectly affect the processes underlying PrP conversion either by stabilizing PrP^{C}-PrP^{Sc} interaction (octarepeats) or by inducing structural (tertiary) changes in PrP^{C} (glycosaminoglycan binding and amyloidogenic motif) facilitating subsequent conversion of PrP. Direct involvement in (di or-) trimerization of PrP^{C} is implicated for the two binding domains [102-WNK-104] and [225-SQAY-228]. This self-interaction precedes partial unfolding and/or refolding into an intermediate form of PrP (PrP^{i}) after which binding to PrP^{Sc} occurs resulting in subsequent conversion. Therefore these two domains may prove prime targets for development of new therapeutic strategies. Taken together the results of this study further emphasize the importance of the stability of the PrP^{C}-PrP^{C} and PrP^{C}-PrP^{Sc} interactions in PrP conversion, which probably is at the basis of the effects of disease associated mutations, as well as the species-barrier. Increased understanding

of the processes involved in PrP conversion may lead to the conception of therapeutic strategies with the possibility to leave the physiological function of the prion protein unaffected.

During the progression of this thesis, technical limitations prevented further pursuing of the intended line of investigation described in project-proposal "*Studies towards the development of therapeutics to treat and/or prevent prion diseases*" granted by The Netherlands Organization for Scientific Research (NWO). We therefore changed to a more fundamental approach, focusing on PrP^{C} itself and aiming to elucidate which amino acid residues of PrP^{C} are involved in PrP interaction and how they affect the processes underlying PrP^{C} to PrP^{Sc} conversion. In order to place our findings in "the larger picture" we reviewed literature (chapter 7) in order to "distill" clues on the physiological function of PrP^{C} from a seemingly surplus of scattered, but detailed studies; focusing more on similarities than details, a more general context of the physiological function of PrP^{C} seems to emerge. A better understanding of the physiological function will have implications on the way future therapeutic strategies will be devised or how current strategies may be improved. This chapter summarizes results on PrP interactions and the contribution the presented research may have in the quest for new or improved therapeutic strategies. Finally the chapter is completed with an overview of the "current state of art" concerning therapy development and testing of promising therapeutic compounds.

Within the scope of the original objective of the project a possible delivery vector for expression of heterelogous PrP^{C} as a therapy was investigated. In parallel down regulation by antisense knockdown of PrP^{C} expression was investigated, even PrP^{C} ablation may not be the ideal approach, considering the consequential loss of function. However, alternative delivery (i.e. using PRV) can be devised as such that knock down of PrP^{C} is reversible instead of indefinite. In chapter 7 the preliminary results hereof have been mentioned as alternatives to lentiviral delivery and siRNA/shRNA downregulation of PrP^{C}. However the review did not allow for an at length discussion of our preliminary data and therefore a more detailed discussion follows.

Lentiviral delivery vectors have been used in experimental prion therapy [27-32], either expressing heterologous PrP^{C}, a soluble PrP antagonist or RNA interference (RNAi) constructs to down-regulate endogenous PrP^{C} expression. Lentiviral delivery is only effective when injected intra-cerebrally (lentivirus cannot pass blood-brain barrier) and delivery is limited to the site of injection. Lentivirus also has the propensity to integrate in the host genome, making therapy using lentiviral delivery irreversible [33]. These properties of lentivirus are less desirable when devising therapeutic therapies that

temporarily interfere with PrP^C expression or PrP (self-) interaction, which may prove vital to restoring PrP^C functionality after treatment. Especially since PrP^C functionality seems to play a pivotal role in pathways that lead to differentiation of particular cells or protection of particular cells by preventing apoptosis (as reviewed in chapter 7) normal PrP^C functionality might need to be retained or restored during anti-prion therapy to prevent (neurological) problems later on. An alternative for lentiviral delivery may be pseudorabies virus (PRV) and the feasibility of PRV as a delivery vector to neuronal tissues was investigated. The rational for using PRV, is the natural tropism of this virus for non-dividing (neuronal and lymphoreticular cells) cells [34]. This also means that direct injection into the brain is not necessary, as is the case when using lentiviral delivery, as PRV is capable of crossing the blood brain barrier. Furthermore viral spread between individuals by PRV can be abolished by knockout of glycoprotein D (gD), which does not affect its neuronal spread [35-39]. Additionally (if necessary) glycoprotein E (gE) can be knocked out to reduce virulence [34] and impair neuronal spread to second order neurons [40, 41], which may prove useful when widespread neuronal spread is not essential whereas additional (over)expression of heterologous conversion resistant PrP may be. Knocking out both glycoprotein D and E by inserting a PrP open reading frame (ORF) should allow for increased PrP expression whilst viral spread of the delivery vector between animals is abolished and the virulence of the delivery vector impaired. There is evidence that heterologous PrP^C is sufficient to stop PrP^{Sc} formation when scrapie-resistant 171R-PrP^C (ARR) is co-expressed in cell culture and mice [12]. Several cases of naturally infected sheep with (homologous) 171R-PrP^C phenotype have been described [42-44] as well as some cases of experimental infection of sheep with the 171R-PrP^C phenotype with BSE [43, 45]. However, resistance to conversion may not have to be complete, but merely sufficiently reduced for therapy to be successful. Furthermore, recently a novel polymorphism in PrP^C has been described that seems to modulate PrP^{Sc} deposition in placentas of naturally and experimentally scrapie-affected ARQ/ARQ sheep [46] and other mutations in PrP can be devised and tested and may prove (completely) conversion resistant. These mutations can be devised to disrupt certain traits of PrP^C like glycosylation or formation of tertiary structures (α-helix, β-sheet), aided by the interaction domains we have peptide-mapped [47] and the involvement of these domains on conversion [48].

In order to provide a "proof of principal" we investigated the ability of PRV to express exogenous protein, the natural scrapie resistant PrP^C isoform 171R. Furthermore 171R-PrP^C potentially can be used to suppress PrP^{Sc} formation. First the open reading frame (ORF) of 171R-PrP including its promoter was cloned in-frame in both glycoprotein D and E of PRV. Insertion of PrP simultaneously resulted in deletion of 146 bp and

336 bp of gD and gE coding sequence respectively, in order to prevent the possibility of the PrP-PRV to lose the PrP-ORF and reverting to wild type. A principle test was conducted by inserting gE-PrP^{171R} into PRV via homologous recombination, resulting in $^{\Delta gE}$PRV-PrP^{171R} delivery vector. Two 96-well plates containing possible reassortant virus clones of PRV were tested by immuno-peroxidase monolayer assay (IPMA) using either a PRV gD monoclonal antibody (Fig.1A) or a PrP specific polyclonal antiserum (Fig.1B). The resulting IPMA screening resulted in 46% of the clones strongly (including 7% weakly) positive for both gD and PrP, 13% only positive for gD and 41% negative (including 1% unclear). This shows that the PrP-ORF can successfully be integrated in the PRV genome and result in (sufficient) expression of PrP^{C}. These preliminary results indicate that PRV is suitable for expression of exogenous protein. However the usability of PRV as a delivery vector to neuronal tissues needs to be further investigated.

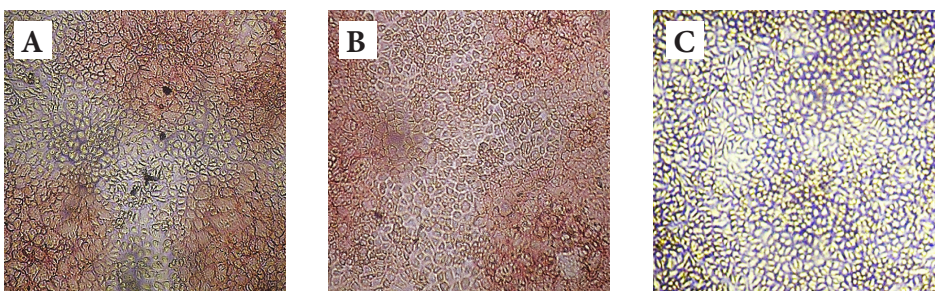


Figure 1. ***IPMA staining of SK6 cells infected with recombinant PRV virus.*** *PRV was modified by homologous recombination of gE, resulting in gE knockout and insertion of the PrP ORF. The obtained recombinant PRV virus was capable of expressing 171R PrP^{C}. Presence of PRV virus was determined by IPMA staining of infected cells using gD specific MAb 45.32.2b (A) and PrP^{C} expression was determined by IPMA staining of infected cells with polyclonal antibody R521 (B). Positive staining was determined by comparing infected cells to non-infected cells (C).*

Parallel to the study of expressing exogenous protein (PrP^{171R}) by PRV we also investigated the feasibility of expressing antisense PrP in order to down-regulate PrP^{C} expression. This approach could be used as a more general approach to knockdown PrP^{C}, possibly offering a broader applicability than recent developments in the field of RNA interference (RNAi). These developments offer a potential means to (temporarily) silence PrP^{C} expression (reviewed in [49]) using specific siRNA or shRNA. Results obtained with transgenic mice deficient for PrP^{C} ($PrP^{0/0}$) showed that PrP^{C} is a prerequisite

for prion (agent) propagation [50, 51]. Therefore, down-regulation of PrP^C may be a straightforward method in preventing disease development. However, resorting to transgenic modification as a therapeutic strategy is quite drastic.

RNAi is a naturally occurring mechanism for post-transcriptional gene silencing in eukaryotes, which is highly conserved and sequence-specific. Normally the open reading frame (ORF) of PrP would be transcribed into messenger RNA (mRNA, Fig.2, a) in the nucleus, which is translocated to the cytoplasm where it is recognized by a ribosome (Fig.2, b) of the endoplasmic reticulum (ER). The ribosome translates the mRNA forming the amino acid backbone of the protein (Fig.2, c) and in the ER post-translation modifications are made (i.e. sulfide-bridges) and the protein is folded (Fig.2, d). When antisense RNA (asRNA) is produced (Fig.2, e), under the right conditions and concentrations, the asRNA anneals to the corresponding mRNA, resulting in double stranded RNA (Fig.2, f). This dsRNA initiates RNA interference, resulting in quick degradation of the dsRNA into 21-23 basepare long double stranded fragments by the enzyme DICER (Fig.2, g). These double stranded RNA fragments are loaded onto the Argonaute protein which is integrated into the multi-protein RNA induced silencing complex or RISC (Fig.2, h). Within RISC the sense strand is removed, leaving the antisense or interfering RNA strand fragment (siRNA). Alternatively, siRNA or short hairpin RNA (shRNA) can be expressed experimentally after which DICER cleaves shRNA into siRNA like oligo's which like siRNA can be integrated in RISC by Argonaute (Fig2, h). Subsequently protein expression is inhibited by siRNA directed annealing of RISC to free mRNA (Fig.2, 1), resulting in "slicing" of the mRNA (Fig.2, 2). Alternatively, siRNA directed annealing of RISC to mRNA during translation (Fig.2, 3) results in 'blocking' of the ribosome, disrupting translation and as a consequence protein expression (Fig.2, 4).

Previously the ORFs of several isoforms of PrP^C (disease susceptible 136V, wildtype ARQ, conversion resistant 171R) were cloned in the eukaryotic expression vector pIHC, which were used to obtain CHO cells stably expressing the different PrP^C isoforms [4]. The ORFs had been cloned into pIHC using the enzymes *Not*I and *Xba*I, which allowed for directional and in-frame cloning. This cloning strategy has left the digestion-sites intact, allowing excision of the PrP ORFs, leaving the promotor, the intron and the polyA tail intact. To obtain an antisense PrP construct in pIHC, the original pIHC-PrP plasmids were digested with *Not*I and *Xba*I and the PrP-ORF and the plasmid pIHC were agarose gel-purified. Into the digested and purified pIHC a linker was ligated that reversed the order of the original *Not*I and *Xba*I sites.

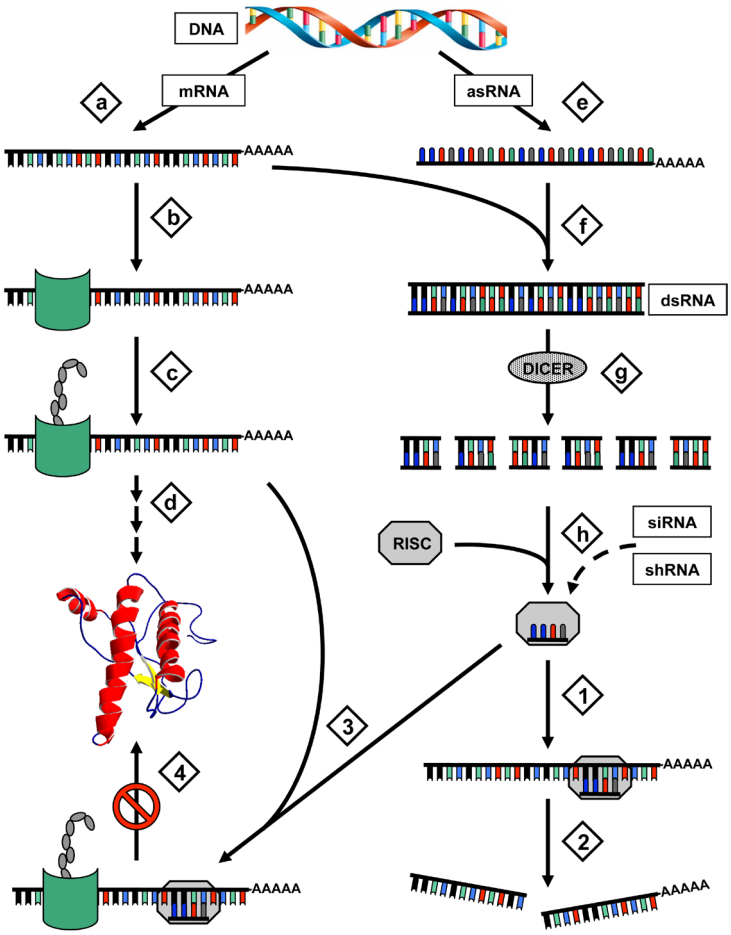


Figure 2. ***Schematic overview of DNA transcription/translation and downregulation by RNAi.*** *The open reading frame (ORF) of PrP is transcribed into messenger RNA (mRNA, a) in the nucleus, translocated to the cytoplasm and recognized by a ribosome (b) of the endoplasmic reticulum (ER). The ribosome translates mRNA attaching amino acid to each other according to the mRNA sequence (c) and in the ER post-translation modifications are made (i.e. sulfide-bridges) and the protein is folded (d). When antisense RNA (asRNA) is produced (e), under the right conditions and concentrations, the asRNA anneals to the corresponding mRNA, resulting in double stranded RNA (f). This dsRNA initiates RNA interference, resulting in quick degradation of dsRNA by DICER (g). These dsRNA fragments are loaded onto the Argonaute protein which is integrated into the multi-protein RNA induced silencing complex (h). Within RISC the sense stand is removed, leaving the antisense or interfering RNA strand fragment (siRNA). Subsequently protein expression is inhibited by siRNA directed annealing of RISC to free mRNA (1), resulting in "slicing" of the mRNA (2) or by siRNA directed annealing of RISC to mRNA during translation (3) 'blocking' the ribosome, disrupting translation and as a consequence protein expression (4).*

This 'reversed' pIHC was again digested using *Not*I and *Xba*I and the original PrP-ORF was directionally ligated back into this vector, resulting in a pIHC containing the PrP-ORF in reverse complement (antisense) orientation to the promoter (pIHC-asPrP).These new pIHC-asPrP constructs were then sequenced to ensure the correct orientation (reverse complement) of the PrP-ORF to the promoter, which should result in an antisense transcription product (asRNA) of PrP.

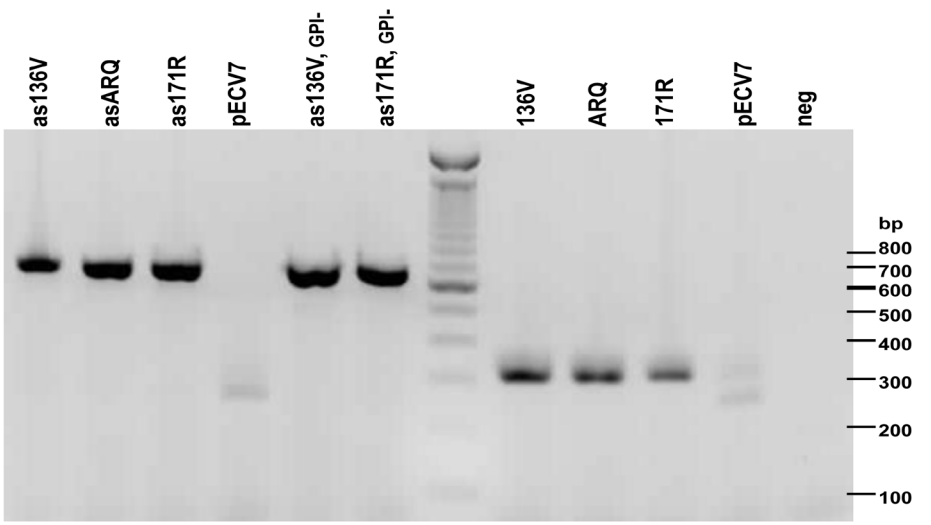


Figure 3. ***Multiplex RT-PCR distinguishing between 'sense' and 'antisense' mRNA.*** *Total RNA was isolated from CHO cells transiently expressing the antisense product of 3 different full length reverse complement variants of PrP (as136V, asVRQ, as171R) and 2 reverse complement variants of lacking the protein anchor sequence GPI (as136V-GPI-, as171R-GPI-). Total RNA was also isolated from the stable cell lines expressing 3 variants of PrP (136V, ARQ, 171R). A multiplex OneStep® RT-PCR was performed in which 'sense' RNA yields a band of 313bp and 'antisense'yields a band of 663bp. As a negative control empty vector pECV7 was transiently expressed or no RNA was added (neg).*

To test if indeed antisense mRNA was formed the construct were electroporated into the CHO cells and then kept in culture to allow expression of (m)RNA. Total RNA was isolated and a multiplex OneStep RT-PCR performed to distinguish between "sense" (313bp) and "antisense" (RNA present in the sample) (Fig.3). All antisense constructs were expressed in N2a CHO cells and resulted in amplification of the correct size OneStep RT-PCR product (Fig.3, lane 1, 2, 3, 5, 6) as compared to the stable cell lines

(Fig.3, lane 8, 9, 10). The negative control (no RNA template) produced no product in the OneStep RT-PCR (Fig.3, lane 12), whereas the control transfection containing only empty vector (PECV7) resulted in a low intensity OneStep RT-PCR product of a size somewhat smaller than the sense product. This is probably due to low expression of endogenous PrP by the N2a cells (Fig.3, lane 4, 11).

Initial co-expression experiments of the antisense constructs in the stable PrP-expressing cell lines were performed. Protein expression was analyzed by SDS-PAGE and Western blotting and preliminary results show a general down regulation of approximately 50% irrespective of the PrP variants tested. This suggests that only specific (identical in all variants) dsRNA fragments are capable of down regulating protein expression. Furthermore it seems that the RNA interference system has limited ability to down regulate protein expression with a maximum down regulation of around 50%, which is comparable to down-regulation achieved with siRNA and shRNA. However, further experiments need to be carried out to confirm these preliminary results.

Concluding remarks.

It is crucial (when possible) to develop strategies against prion disease that are effective not only as a prophylactic but also when administered in the (pre-) clinical phase, when disease related neurological symptoms have developed. Ideally newly developed therapies allow for retention or restoration of normal physiological functionality of the target (PrP^{C}). Accomplishing such a therapy necessitates study of several aspects of the prion protein and prion disease; understanding the physiological function of PrP^{C}, elucidating the processes underlying disease development (conversion), development/improvement of diagnostic tools (providing early pre-clinical diagnosis) and development of specific and effective therapeutics or therapeutic strategies.

In order to understand the molecular mechanism of conversion it is imperative to know which amino acid sequences (domains) are involved in interaction between PrP^{C} molecules (self-interaction), between PrP^{C} and PrP^{Sc} and/or in conversion of PrP^{C} into PrP^{Sc}. This knowledge should allow for a more focused approach in therapy development. At the same time PrP^{C} function needs to be elucidated in order to circumvent possible drawbacks; knowing PrP^{C} function will allow better assessment of possible (long term) harmful effects of therapy and whether the severity of the advantages outweigh the disadvantages. To improve diagnostic tools for TSEs and allow for earlier intervention,

increased understanding of the molecular processes of conversion and physiological function of PrP^{C} can only be taken fully advantage of when equal effort is invested in both conditions. Together all these improvements may eventually lead to preclinical or early diagnosis that allows for earlier implementation of existing therapies (improving affectivity) or newly devised therapies against specific PrP interactions. Ultimately leading to a therapeutic (strategy) not only capable of halting conversion of PrP^{C} into PrP^{Sc}, but also capable of clearing already present PrP^{Sc} providing the necessary conditions that may allow for reversion of (early) neurological symptoms.

Recently Aguzzi noted compelling evidence of intriguing similarities between the prion principle and amyloidoses in which proteins are misfolded and aggregated into various harmful forms [52]. Mammalian amyloidoses like Alzheimer's disease, Tauopathies, Parkinson's disease and Huntingting's disease share the potential to molecularly transmit (self-sustaining aggregation). However these amyloidoses termed 'prionoids' do not behave like typical infectious agents and do not evoke a complete infectious life cycle like prions. Prion diseases are transmissible between individuals and often across species whereas prionoids are not. However, a possible exception may be AA amyloidosis, which seems to display all the elements of a complete infectious cycle (including uptake, replication and release from its host). If these similarities between these amyloid diseases are indicative for common basic principles in neurodegenerative diseases forming amyloid and non-amyloid protein-depositions (for a review see also [53]), adequate knowledge of the (molecular) processes underlying transmissible spongiform encephalopathies could possibly lead to using TSEs and the developed techniques for study of these amyloid diseases. TSEs may be used as a model to study more general and common molecular mechanisms making use of the infectious and self-propagating properties. This may lead to new and formerly unattainable information on neurodegenerative diseases other than TSEs, providing a new (supplemental) scope for studying these neurodegenerative diseases. As prion disease occurs as a hereditary, sporadic and infectious disease, comparisons between these different forms of TSE should provide clues to which molecular mechanisms are specific for infectivity. Therefore, the common denominators between neurodegenerative diseases can be investigated more intensively by investigating protein misfolding mechanisms in transmissible spongiform encephalopath.

References.

1. Griffith, J.S., *Self-replication and scrapie.* Nature, 1967. 215(105): p. 1043-4.
2. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. 216(4542): p. 136-44.
3. Bossers, A., *Prion Disease: Susceptibility and Transmissibility; In vivo and in vitro studies with sheep scrapie*. 1999, University of Utrecht.
4. Bossers, A., et al., *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4931-6.
5. Bossers, A., R. de Vries, and M.A. Smits, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP.* J Virol, 2000. 74(3): p. 1407-14.
6. Caughey, B., et al., *Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state.* Chem Biol, 1995. 2(12): p. 807-17.
7. Caughey, B., et al., *Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies.* J Virol, 1997. 71(5): p. 4107-10.
8. Raymond, G.J., et al., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo J, 2000. 19(17): p. 4425-30.
9. Raymond, G.J., et al., *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans.* Nature, 1997. 388(6639): p. 285-8.
10. Holscher, C., H. Delius, and A. Burkle, *Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation.* J Virol, 1998. 72(2): p. 1153-9.
11. Chabry, J., B. Caughey, and B. Chesebro, *Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides.* J Biol Chem, 1998. 273(21): p. 13203-7.
12. Horiuchi, M., et al., *Inhibition of interactions and interconversions of prion protein isoforms by peptide fragments from the C-terminal folded domain.* J Biol Chem, 2001. 276(18): p. 15489-97.
13. Peretz, D., et al., *A conformational transition at the N terminus of the prion protein features in formation of the scrapie isoform.* J Mol Biol, 1997. 273(3): p. 614-22.
14. Beringue, V., et al., *PrPSc binding antibodies are potent inhibitors of prion replication in cell lines.* J Biol Chem, 2004. 279(38): p. 39671-6.
15. Gasset, M., et al., *Perturbation of the secondary structure of the scrapie prion protein under conditions that alter infectivity.* Proc Natl Acad Sci U S A, 1993. 90(1): p. 1-5.

16. Heppner, F.L., et al., *Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies.* Science, 2001. 294(5540): p. 178-82.
17. Kocisko, D.A., et al., *Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier.* Proc Natl Acad Sci U S A, 1995. 92(9): p. 3923-7.
18. Korth, C., et al., *Prion (PrPSc)-specific epitope defined by a monoclonal antibody.* Nature, 1997. 390(6655): p. 74-7.
19. Peretz, D., et al., *Antibodies inhibit prion propagation and clear cell cultures of prion infectivity.* Nature, 2001. 412(6848): p. 739-43.
20. Perrier, V., et al., *Anti-PrP antibodies block PrPSc replication in prion-infected cell cultures by accelerating PrPC degradation.* J Neurochem, 2004. 89(2): p. 454-63.
21. Priola, S.A. and B. Chesebro, *A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells.* J Virol, 1995. 69(12): p. 7754-8.
22. Priola, S.A. and V.A. Lawson, *Glycosylation influences cross-species formation of protease-resistant prion protein.* Embo J, 2001. 20(23): p. 6692-9.
23. Scott, M., et al., *Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes.* Cell, 1993. 73(5): p. 979-88.
24. Bossers, A., et al., *In vitro conversion of normal prion protein into pathologic isoforms.* Clin Lab Med, 2003. 23(1): p. 227-47.
25. Govaerts, C., et al., *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A, 2004. 101(22): p. 8342-7.
26. Langedijk, J.P., et al., *Two-rung model of a left-handed beta-helix for prions explains species barrier and strain variation in transmissible spongiform encephalopathies.* J Mol Biol, 2006. 360(4): p. 907-20.
27. Crozet, C., et al., *Inhibition of PrPSc formation by lentiviral gene transfer of PrP containing dominant negative mutations.* J Cell Sci, 2004. 117(Pt 23): p. 5591-7.
28. Genoud, N., et al., *Antiprion prophylaxis by gene transfer of a soluble prion antagonist.* Am J Pathol, 2008. 172(5): p. 1287-96.
29. Golding, M.C., et al., *Suppression of prion protein in livestock by RNA interference.* Proc Natl Acad Sci U S A, 2006. 103(14): p. 5285-90.
30. Pfeifer, A., et al., *Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice.* J Clin Invest, 2006. 116(12): p. 3204-10.
31. Toupet, K., et al., *Effective gene therapy in a mouse model of prion diseases.* PLoS ONE, 2008. 3(7): p. e2773.
32. White, M.D., et al., *Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease.* Proc Natl Acad Sci U S A, 2008. 105(29): p. 10238-43.

33. Nayak, S. and R.W. Herzog, *Progress and prospects: immune responses to viral vectors.* Gene Ther. 17(3): p. 295-304.

34. Tirabassi, R.S., et al., *Molecular mechanisms of neurotropic herpesvirus invasion and spread in the CNS.* Neurosci Biobehav Rev, 1998. 22(6): p. 709-20.

35. Mulder, W., et al., *Glycoprotein D-negative pseudorabies virus can spread transneuronally via direct neuron-to-neuron transmission in its natural host, the pig, but not after additional inactivation of gE or gI.* J Virol, 1996. 70(4): p. 2191-200.

36. Peeters, B., et al., *Biologically safe, non-transmissible pseudorabies virus vector vaccine protects pigs against both Aujeszky's disease and classical swine fever.* J Gen Virol, 1997. 78 (Pt 12): p. 3311-5.

37. Peeters, B., et al., *Glycoprotein H of pseudorabies virus is essential for entry and cell-to-cell spread of the virus.* J Virol, 1992. 66(6): p. 3888-92.

38. Peeters, B., et al., *Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion.* J Virol, 1992. 66(2): p. 894-905.

39. Peeters, B., et al., *Envelope glycoprotein gp50 of pseudorabies virus is essential for virus entry but is not required for viral spread in mice.* J Virol, 1993. 67(1): p. 170-7.

40. Ferrari, M., et al., *A comparative study of pseudorabies virus (PRV) strains with defects in thymidine kinase and glycoprotein genes.* J Comp Pathol, 2000. 123(2-3): p. 152-63.

41. Mettenleiter, T.C., *Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis--state of the art, June 1999.* Vet Res, 2000. 31(1): p. 99-115.

42. Buschmann, A., et al., *Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR).* J Gen Virol, 2004. 85(Pt 9): p. 2727-33.

43. Groschup, M.H., et al., *Classic scrapie in sheep with the ARR/ARR prion genotype in Germany and France.* Emerg Infect Dis, 2007. 13(8): p. 1201-7.

44. Ikeda, T., et al., *Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan.* J Gen Virol, 1995. 76 (Pt 10): p. 2577-81.

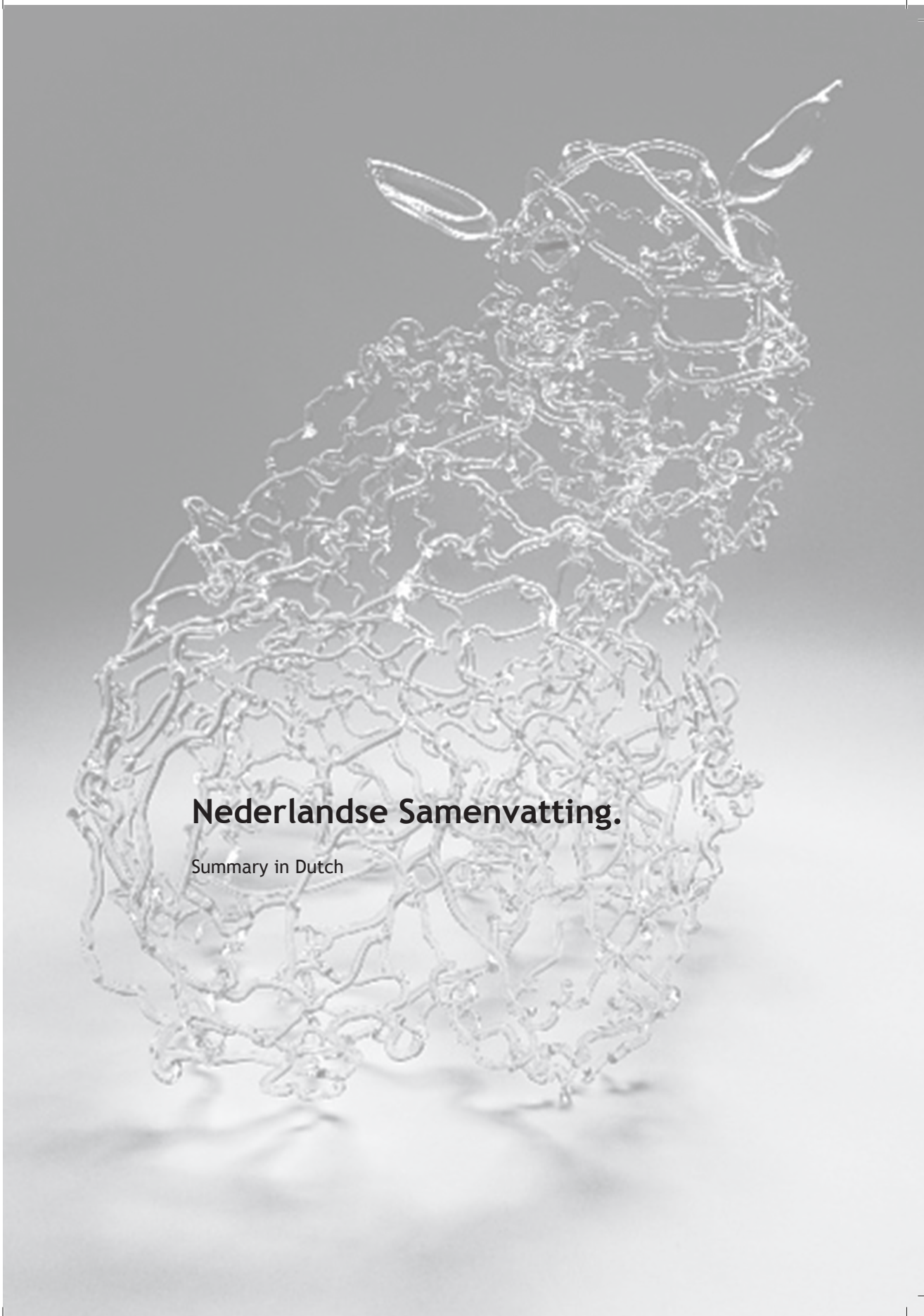
45. Jeffrey, M., et al., *Onset and distribution of tissue prp accumulation in scrapie-affected suffolk sheep as demonstrated by sequential necropsies and tonsillar biopsies.* J Comp Pathol, 2001. 125(1): p. 48-57.

46. Santucciu C, M.C., Madau L, Attene S, Cancedda MG, Demontis F, Tilocca MG, Saba M, Macciocu S, Carta A, Ligios C., *Association of N176K and L141F dimorphisms of the PRNP gene with lack of PrPSc deposition in placentas of naturally and experimentally scrapie-affected ARQ/ARQ sheep.* J Gen Virol, 2010. in print.

47. Rigter, A., et al., *Mapping of possible prion protein self-interaction domains using peptide arrays.* BMC Biochem, 2007. 8: p. 6.

48. Rigter, A., et al., *Prion protein self-peptides modulate prion interactions and conversion.* BMC Biochem, 2009. 10: p. 29.

49. Tomari, Y. and P.D. Zamore, *Perspective: machines for RNAi.* Genes Dev, 2005. 19(5): p. 517-29.
50. Bueler, H., et al., *Mice devoid of PrP are resistant to scrapie.* Cell, 1993. 73(7): p. 1339-47.
51. Sailer, A., et al., *No propagation of prions in mice devoid of PrP.* Cell, 1994. 77(7): p. 967-8.
52. Aguzzi, A., *Cell biology: Beyond the prion principle.* Nature, 2009. 459(7249): p. 924-5.
53. Winklhofer, K.F., J. Tatzelt, and C. Haass, *The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases.* Embo J, 2008. 27(2): p. 336-49.



Nederlandse Samenvatting.

Summary in Dutch

Recentelijk is een aandoening in de belangstelling gekomen, waarvan is gebleken dat deze niet wordt veroorzaakt door besmettelijke micro-organismen (bacteriën en schimmels), virussen of parasieten. De verwekkers van deze zeer ernstige en dodelijke aandoening behoren tot de overdraagbare spongiforme encephalopathieën (Transmissible Spongiform Encephalopathies, TSEs), ook wel prionziekten genaamd. Tot de prionziekten behoort een verzameling van voortschrijdende (progressieve) aandoeningen die de weefsels van het centraal zenuwstelsel aantasten. Verschillende vormen van TSEs komen voor bij zowel mens als dier, te weten sporadisch (sporadic Creutzfeldt-Jacob Disease [CJD]), erfelijk (Gerstmann-Sträussler-Scheinker Syndrome, Fatal Familial Insomnia, familial CJD) of infectieus (Bovine Spongiform Encephalopathy ['gekke koeien ziekte', BSE], scrapie, iatrogenic CJD, variant CJD). Het prion eiwit (PrP) is een gastheereigen eiwit, dat voornamelijk in de cellen van het centrale zenuwstelsel (CZS) wordt aangemaakt. Het is onduidelijk op welke manier deze aandoeningen worden veroorzaakt, maar kenmerkend voor het ziekteverloop van TSEs is het hervouwen van de ruimtelijke structuur van het normale gastheereigen eiwit (PrP^{C}) naar een met de ziekte geassocieerde abnormale ruimtelijke structuur van PrP (PrP^{Sc}). Dit 'verkeerd' gevouwen PrP^{Sc} kan daardoor niet (afdoende) opgeruimd worden door eiwitafbrekende enzymen waardoor het ophoopt in de cellen van het CZS. Het gevolg van het ophopen van PrP^{Sc} is dat neuronen (hersen –en zenuwcellen) degenereren, wat leidt tot sponsvorming van het CZS-weefsel. De manier waarop de ruimtelijke structuur van het PrP wordt veranderd is tot op heden onduidelijk. In dit proefschrift wordt onderzoek naar factoren die het hervouwing proces beïnvloedden beschreven. Dit leidde tot het onderzoeken naar welke 'delen' van het PrP eiwit en op wat voor manier deze betrokken zijn in de verschillende onderliggende processen die tot hervouwing leiden. Door de betrokken specifieke processen in kaart te brengen, kunnen mogelijkerwijs huidige behandelmethoden (therapieën) verbeterd worden en wellicht vernieuwende therapieën ontwikkeld worden.

Tot op heden geldt de hypothese dat PrP^{Sc} het infectieuze deeltje in prionziekten is. Behandeling met middelen die genetisch materaal (DNA, RNA) afbreken hebben geen effect op het infectieuze karakter van prionziekten. Hieruit bleek dat prionziekten uniek zijn en niet verwekt worden door de 'bekende' ziektewekkers, maar door een infectieus eiwit. PrP^{Sc} kan zijn driedimensionale structuur 'opleggen' aan normaal PrP^{C}, waardoor nieuwe infectieuze deeltjes ontstaan (ofwel prion/agens replicatie) en deze ruimtelijke structuurveranderingen (hervouwing) wordt ook wel conversie genoemd. Het nieuw gevormde infectieuze deeltje is opgebouwd uit dezelfde bouwstenen (aminozuren) als het PrP^{C} waaruit het is ontstaan (primaire structuur). Echter, de verhoudingen/ aanwezigheid van de driedimensionale eiwit-structuren (α-helix en β-sheet) zijn

veranderd (secundaire structuur). Als gevolg hiervan is ook de ruimtelijke ordening van het gehele eiwit veranderd (tertiaire structuur), met als gevolg een aantal biochemische verschillen tussen de verschillende 'vouwingen' van het prioneiwit.

De eerste gedocumenteerde gevallen van scrapie in schapen zijn al beschreven in 1732 en het infectieuze karakter van scrapie bij schapen werd aangetoond in 1936. Dit maakt scrapie de best beschreven, natuurlijk spreidende en langst bestudeerde TSE en daarom een zeer geschikte kandidaat om als model te fungeren voor TSE onderzoek. Bij aanvang was het bekend dat de aanwezigheid van het scrapie agens een voorwaarde is voor de ontwikkeling van scrapie. Tevens was bekend dat verschillen in het PrP-gen van de gastheer (polymorfismen, allel-varianten, genotype) van invloed zijn op de gevoeligheid voor scrapie en het ziekteverloop in de gastheer (fenotype). Verschillen in het PrP-gen tussen verschillende (dier-)soorten (species) zijn van invloed op de overdraagbaarheid van PrP^{Sc} tussen soorten, ofwel de gevoeligheid van een gastheer voor infectieuze deeltjes afkomstig van een andere soort. Deze verschillen in gevoeligheid en overdraagbaarheid tussen species wordt ook wel de soort-barrière genoemd.

Bij aanvang van het onderzoek was een celvrije *in vitro* (in een reageerbuis) syteem beschikbaar dat kon worden gebruikt om de omvorming van PrP^{C} naar PrP^{Sc} te meten (conversie). De conversie-efficiënties van verschillende schapen PrP^{C}-varianten in dit *in vitro* conversie systeem zijn representatief voor de gevoeligheid van de gastheer voor het ontwikkelen van scrapie. Tevens was dit systeem gebruikt om de overdraagbaarheid van TSEs tussen verschillende soorten te bestuderen. De efficiëntie van omzetting door PrP^{Sc} van een andere species van het gastheer PrP^{C} is indicatief voor de overdraagbaarheid van de betreffende TSE naar de gastheer (soort-barrière), bijvoorbeeld de overdraagbaarheid van BSE (van koeien) naar de mens. Dit geeft echter ook aangrijpingsmogelijkheden om conversie te remmen of zelfs te stoppen en zodoende prionziekten te voorkomen (preventie) of te behandelen (therapie).

Echter, het exacte proces van hervouwing (de moleculaire mechanismen) is nog steeds een enigma. Zo ook het mechanisme achter de verschillen in conversie-efficiëntie ten gevolge van de PrP^{C} polymorfismen. De studies beschreven in dit proefschrift zijn er op gericht om deze moleculaire mechanismen gedetailleerder in kaart te brengen. De eerste stap in het conversie (hervouwings) proces is het binden van PrP^{C} aan PrP^{Sc}. Daarom werd voor dit proefschrift als eerste onderzocht of de polymorfismen in schapen PrP van invloed zijn op de efficiëntie waarmee PrP^{C} aan PrP^{Sc} bind en of dit gecorreleerd kan worden aan de verschillen in conversie-efficiëntie van de PrP-varianten (hoofdstuk 3). Deze studie liet zien dat het binden van verschillende PrP^{C} varianten aan

verschillende PrP^{Sc} varianten PrP specifiek, zeer efficiënt en vergelijkbaar is voor alle combinaties. Hieruit kon geconcludeerd worden dat de conversie efficiëntie verschillen voor de gemeten schapen varianten niet bepaald worden door de initiële PrP^{C}-PrP^{Sc} binding efficiëntie, maar dat de verschillen in conversie-efficiëntie een gevolg zijn van intrinsieke eigenschap(pen) van het PrP^{C} zelf. Deze intrinsieke eigenschap(pen) van PrP^{C} wordt beïnvloed door de polymorfismen in PrP. Dit betekent dat er een tweede stap (of meerdere stappen) na PrP^{C}-PrP^{Sc} binding verantwoordelijk is voor de geobserveerde verschillen in conversie-efficiëntie.

Het meest logische vervolg van deze resultaten was om te onderzoeken of conversie-resistente (natuurlijk of artificieel) PrP-varianten gebruikt kunnen worden om PrP conversieproces te verstoren en conversie significant kunnen vertragen of blokkeren. Zo zou de conversie-resistente variant bestudeerd kunnen worden als mogelijke therapie voor TSEs. Echter, het celvrije *in vitro* conversie systeem bleek niet robuust genoeg om subtiele verschillen in conversie-efficiëntie te meten, wat het vervolgonderzoek hinderde. Aangezien de precieze moleculaire processen die ten grondslag liggen aan conversie nog steeds onduidelijk waren is besloten om eerst een meer fundamentele aanpak te volgen om beter inzicht en daarmee meer aangrijpingspunten op het conversiemechanisme te krijgen.

Uit eerder onderzoek is aannemelijk gebleken dat interacties tussen PrP^{C} moleculen onderling (zelf-interactie) en tussen PrP^{C} en PrP^{Sc} aan de basis staan van de geobserveerde verschillen in conversie-efficiëntie. Mogelijk zijn er ook interacties met andere eiwitten (co-factoren), die de verschillen in conversie-effeciëntie beïnvloeden. Uit modellen die de (mogelijke) structuur van PrP^{Sc} complexen/aggregaten beschrijven is gebleken dat het aannemelijk is dat PrP^{C} moleculen eerst interactie met elkaar aangaan en een molecuul-complex vormen, de zogenaamde oligomerisatie van PrP^{C} moleculen. Tijdens de oligomerisatie en/of na binding met PrP^{Sc} vindt (gedeeltelijke) ontvouwing van PrP plaats, voordat de uiteindelijke hervouwing naar de ruimtelijke structuur van PrP^{Sc} plaatsvindt. Het in kaart brengen van de specifieke aminozuren van het PrP eiwit die in staat zijn om (zelf-) interactie aan te gaan, zou inzicht moeten geven in welke aminozuren of aminozuur-sequenties (domeinen) van invloed zijn op binding en de daaropvolgende conversie van PrP^{C}. Hiervoor is gebruik gemaakt van peptide-arrays bestaande uit elkaar overlappende en opvolgende peptiden van elk 15 aminozuren. Samen zijn alle peptiden representatief voor de gehele aminozuur-sequentie (volgorde) van het schapen of runder PrP eiwit. Door PrP te laten binden aan deze peptide-array zijn de aminozuur-sequenties die in staat zijn interactie/binding aan te gaan systematisch in kaart gebracht (hoofdstuk 4). Deze studie identificeerde zeven specifieke bindingdomeinen en toonde aan dat

meerdere PrP specifieke zelf-interacties mogelijk zijn tussen PrP^{C} moleculen, evenals tussen PrP^{C} en PrP^{Sc}. Deze specifieke interacties worden waarschijnlijk beïnvloed door verschillen in PrP, uiteindelijk resulterend in verschillen in soort-barrière en tussen PrP^{Sc} 'stammen'.

Selectieve zelf-interactie tussen PrP moleculen staat aan de basis van de geobserveerde verschillen in hervouwing van PrP^{C} door PrP^{Sc} tijdens het conversieproces. Echter, de precieze processen waarin ieder van de in kaart gebrachte interactie domeinen een rol spelen en de effecten hiervan op binding en conversie waren nog onduidelijk. Om dit te achterhalen werden de effecten van zes PrP peptiden op PrP binding aan de peptide-array bestudeerd. Tegelijkertijd werden de effecten van deze peptiden op conversie met behulp van een nieuw en recent ontwikkeld *in vitro* conversie test bestudeerd, na adaptatie van de techniek voor onderzoek naar scrapie (hoofdstuk 6). De "*protein misfolding cyclic amplification*" (PMCA) *in vitro* techniek maakt gebruik van hersen-homogenaten van scrapie-geïnfecteerde en negatieve schapen, waardoor deze techniek de natuurlijke (*in vivo*; binnen het organisme) situatie nauwkeuriger nabootst, aangezien alle mogelijke benodigde co-factoren ook aanwezig zijn in de homogenaten, in tegenstelling tot de eerder beschreven cel-vrije *in vitro* conversietest.

De studie identificeerde het specifieke domein dat vermoedelijk van belang is in de soort-barrière. Dit domein bevond zich in de 'centrale' regio van het PrP^{C} dat al eerder werd aangegeven als het gebied dat bij de soort-barrière betrokken is. Verder werden indicaties gevonden dat de flexibele staart (N-terminale staart) van PrP^{C} de interactie tussen PrP^{C} en PrP^{Sc} stabiliseert met behulp van een zich herhalend domein, beter bekend als de octarepeat. Het uiterste (vrije) puntje van de flexibele staart bevat ook een domein waaraan lange suikerketens ('*glycosaminoglycanen*') kunnen binden, die indirect de conversie-efficiëntie verhogen, wellicht door het faciliteren of verstevigen van de stabilisatie van de PrP^{C}-PrP^{Sc} binding met behulp van de octarepeats. Vlak voor de centrale regio bevat PrP^{C} een domein ('*amyloidogene*' motief) dat op geen enkele manier betrokken lijkt bij PrP binding, maar dat wel indirect de conversie-efficiëntie kan beïnvloeden, waarschijnlijk omdat het de ruimtelijke structuur van de flexibele staart beïnvloed en daarmee de stabilisatie van PrP^{C}-PrP^{Sc} binding.

Twee specifieke domeinen zijn echter direct betrokken in de interactie tussen PrP^{C} moleculen, waarschijnlijk in de vorming van een PrP molecuuol-complex (oligomerisatie) van twee (dimerisatie) en/of drie (trimerisatie) PrP^{C} moleculen. Dit maakt deze twee domeinen de voornaamste doelwitten voor ontwikkeling van nieuwe therapeutische strategieën. De studie benadrukt verder het belang van de PrP^{C}-PrP^{C} en PrP^{C}-Prp^{Sc}

interacties tijdens het hervouwings (conversie-) proces. Het is aannemelijk dat alle interacties een rol spelen in een complex proces waar polymorfismen en de soort-barrière TSE overdraagbaarheid en gevoeligheid beïnvloeden. De effecten van de gebruikte peptiden op conversie konden verklaard worden, maar het kan niet uitgesloten worden dat de peptiden wellicht interactie aangingen met conversie remmende factoren in de hersen-homogenaten die gebruikt werden voor de PMCA. Als resultaat van die interactie kon conversie ongehinderd plaatsvinden, wat de conversie-effeciëntie verhoogde. Het identificeren van deze 'natuurlijke' remmende factoren kan mogelijk een alternatieve onderzoeksrichting zijn naar de moleculaire mechanismen die ten grondslag aan agens replicatie liggen. Dit onderzoek kan mogelijkerwijs verschillende andere doelwitten opleveren die van belang kunnen zijn in het ontwikkelen van toekomstige nieuwe prion therapieën.

Het beschreven onderzoek toont aan dat het moleculaire mechanisme achter het hervouwen van PrP^{C} door PrP^{Sc} zeer complex is en uit verschillende elkaar opvolgende stappen is opgebouwd. De resultaten bevestigen dat het vormen van een complex (oligomerisatie) van meerdere PrP^{C} moleculen (quaternaire structuur) - waarschijnlijk drie (trimerisatie) - zeer belangrijk is in het conversieproces. In het bijzonder de stabiliteit van de PrP^{C}-PrP^{C} en PrP^{C}-PrP^{Sc} interacties zijn van belang voor de efficiëntie waarmee PrP^{C} uiteindelijk geconverteerd kan worden tot PrP^{Sc}. Uit het onderzoek is verder gebleken dat polymorfismen in PrP^{C} het conversieproces indirect beïnvloedden, waarschijnlijk door de invloed van deze polymorfismen op de stabiliteit van de regio in PrP^{C} die verantwoordelijk is voor conversiegevoeligheid en TSE overdraagbaarheid (soort-barrière). Wanneer conversie mogelijk is, is de stabiliteit van de N-terminale staart van het eiwit van belang (stabiliteit bevorderd conversie), waarschijnlijk teweeg gebracht door de interactie tussen de octarepeats en het '*amyloidogene*' motief. Mogelijk speelt deze interactie een rol in de complexvorming van meerdere PrP^{C} moleculen. Verder lijkt er een rol weg gelegd voor de disulfide brug in het C-terminale deel van PrP^{C}, waarbij een stabiele disulfide brug conversie bemoeilijkt. Naast deze intrinsieke eigenschappen van PrP^{C} is het aannemelijk dat glycosaminoglycanen een rol als co-factor in conversie vervullen. Deze lange ketens van suikers (glycosaminoglycanen) kunnen binden aan het domein aan het uiterste N-terminale deel van PrP^{C}, en zodoende mogelijkerwijs de PrP^{C}-PrP^{Sc} interactie faciliteren en/of stabiliseren.

Tot op heden is de functie van het PrP^{C} eiwit nog onbekend, maar dat deze van belang is kan worden afgeleid uit het feit dat sequentie (volgorde van bouwstenen) van het PrP-gen geconserveerd (nauwelijks aan verandering onderhevig) zijn. Daarbij lijkt PrP^{C} een cruciale rol te spelen in signaaltransductie (doorgeven van signalen binnen een cel,

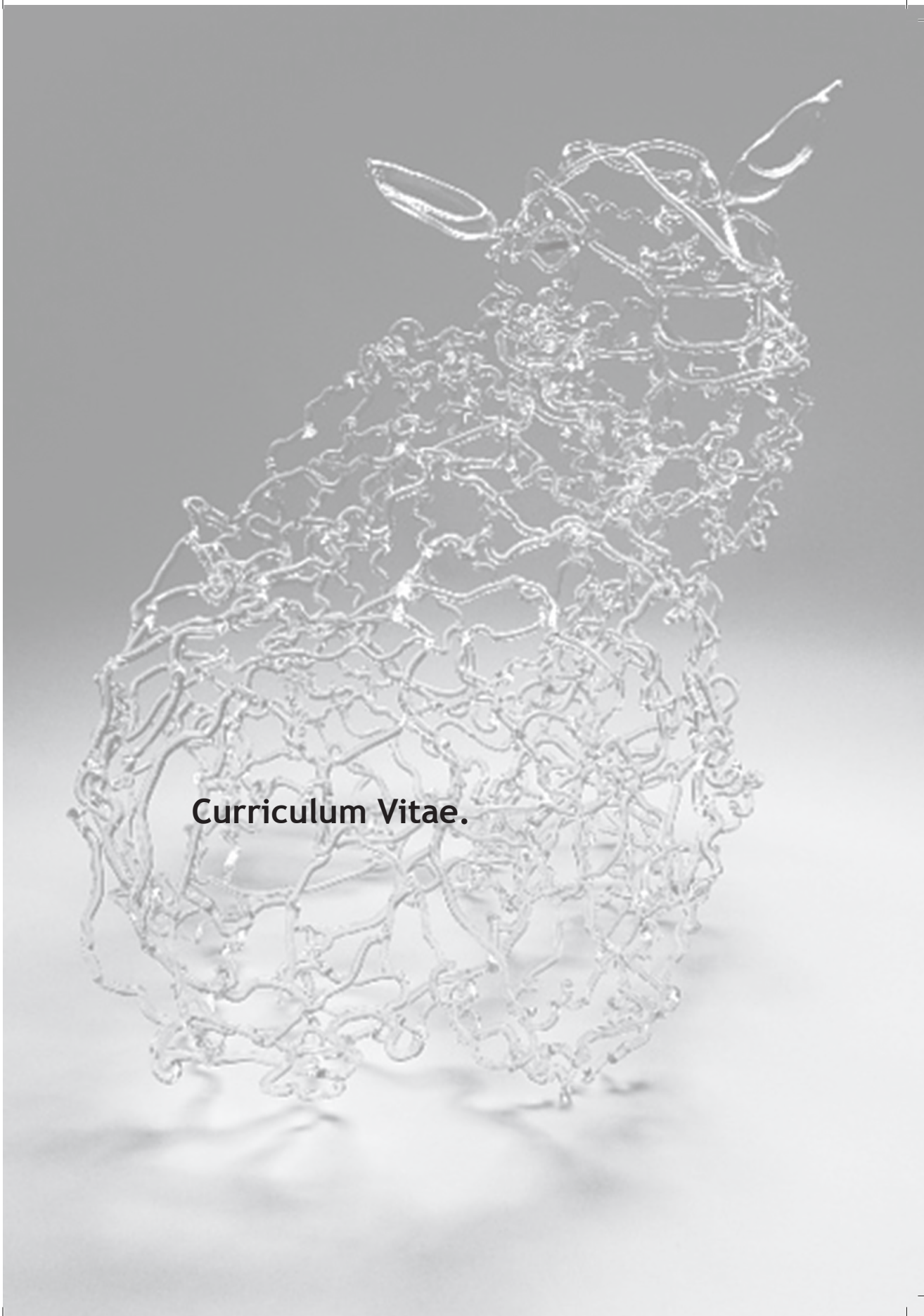
zie hoofdstuk 7), wat van invloed is op het overleven en differentiëren van neuronen. Huidige prionziekten therapieën zijn grofweg in te delen in drie groepen, gebaseerd op het doelwit van de therapie/strategie; op PrP^{C} gerichte therapieën, therapieën gericht op het verhinderen van de PrP^{C}-PrP^{Sc} interactie en op PrP^{Sc} gerichte therapieën (zie hoofdstuk 7).

Aangezien het aannemelijk is dat PrP^{C} een belangerijke functie vervuld, dienen prionziekten therapieën idealiter rekening houden met het waarborgen van PrP^{C} functionaliteit, door de functie van PrP^{C} ongeschonden te laten of te herstellen na behandeling. Aan de hand van het beschreven onderzoek kunnen wellicht nieuwe PrP^{C} gerichte therapieën ontwikkeld worden waarbij de focus ligt op het interfereren in de formatie van PrP^{C} molecuul-complexen (oligomerisatie). Door de vastgestelde zelf-interactie domeinen (tijdelijk) te blokkeren zou oligomerisatie, met als gevolg hervouwing voorkomen kunnen worden terwijl PrP^{C} functie bewaard blijft. Een andere optie zou het (tijdelijk) blokkeren van de domeinen van PrP^{C} die interactie aangaan met PrP^{Sc} zijn, wat mogelijkerwijs ophoping van PrP^{Sc} in de neuronen stopt (vertraagd) waardoor de eiwitafbrekende enzymen de mogelijkheid krijgen om het reeds opgehoopte PrP^{Sc} alsnog op te ruimen.

Tot nu toe zijn vele verschillende therapieën *in vitro* (in een reageerbuis) getest, waarvan verschillende ook *in vivo* (in een gastheer) zijn getest. Vooralsnog zijn de *in vivo* geteste therapieën voornamelijk succesvol wanneer ze preventief worden gebruikt. Dit benadrukt de noodzaak om nieuwe strategieën te ontwikkelen, waarbij nieuwe therapieën worden ontwikkeld (zie hierboven) naast het verbeteren van huidige therapieën (met oog op het behouden van PrP^{C} functie). Onderdeel van de nieuwe strategieën zou het combineren van therapieën kunnen zijn. Bijvoorbeeld door PrP^{C} tijdelijk weg te nemen of door PrP^{C} molecuul-complex vorming te voorkomen [*een op PrP^{C} gerichte therapie*] in combinatie met een therapie die PrP^{C}-PrP^{Sc} interactie voorkomt. Dit zou eventueel nog aangevuld kunnen worden met een therapie die afbraak/opruiming van PrP^{Sc} bevorderd [*een op PrP^{Sc} gerichte therapie*].

Het in dit proefschrift beschreven onderzoek heeft bijgedragen aan een verbeterd inzicht in de moleculaire mechanismen die ten grondslag liggen aan prion-prioneiwit interacties en daarmee de prionconversie. Uit de resultaten is gebleken dat (stabiliserende) zelf-interacties PrP van belang zijn in het conversieproces en dat focussen op deze interacties tot ontwikkeling van nieuwe therapieën voor prionziekten kan leiden. Prionziekten leiden tot ophoping van PrP^{Sc} in neuronen wat uiteindelijk leid tot degeneratie. Het ligt voor de hand dat het verlies van neuronen afbraak doet aan het functioneren van het centraal

zenuwstelsel. Hierdoor kunnen de symptomen van prionziekten verklaard worden. Het is echter niet duidelijk het functioneren van de neuronen alleen geschaad wordt wanneer de neuronen afsterven door PrP^{Sc} ophoping. Het is goed mogelijk dat het functioneren van de neuronen geschaad wordt zodra het normale PrP^{C} weggenomen wordt (en daarmee de functie van PrP^{C}) door hervouwing naar PrP^{Sc}. Wanneer ophoping van PrP^{Sc} tijdig tot een halt wordt geroepen met als gevolg dat de al opgehoopte PrP^{Sc} alsnog wordt opgeruimd, kan herstel van functie (PrP^{C} functie en daarmee het functioneren van het neuronaal weefsel) bewerkstelligd worden. Een effectieve therapie zal dan niet alleen de vooruitgang en symptomen van de prionziekten stoppen, maar zelfs de ommekeer van de symptomen teweeg kunnen brengen. Daarom is het van belang om prion therapieën te ontwikkelen waarbij de functie van PrP^{C} hersteld kan worden of intact blijft, wat mogelijk gefaciliteerd wordt door de verbeterde inzichten in het conversieproces (mede) ten gevolge van het hier gepresenteerde onderzoek.



Curriculum Vitae.

Curriculum Vitae.

Alan Rigter werd geboren op 20 juli 1972 te Amsterdam. Na het behalen van het HAVO diploma in 1989, kwam hij terecht op het Verstveldt Ghijssen Instituut te Utrecht alwaar hij de Klinisch Medische richting van het Middelbaar Laboratorium Onderwijs volgde. Deze opleiding werd afgerond in 1993 na een stage in het klinish-chemisch laboratorium en bij de bloedtransfusiedienst van het Vrije Universiteit (VU) ziekenhuis. Hierna vervolgde hij zijn opleiding aan het Hoger Laboratorium Onderwijs, afstudeer richting Biotechnologie van de Hogeschool van Utrecht, Faculteit Natuur & Techniek. Deze opleiding werd afgerond in 1997 na een stage bij de afdeling Applied Biology and Biotechnology van het Royal Melbourne Institute of Technology (RMIT) in Melbourne Australië. Tijdens deze stage nam hij ook deel aan het Bachelor of Applied Science (Honours) programma, waarvoor hij een equivalent Bachelor of Applied Science degree (Honours Class I) ontving.

Na zijn opleiding begon Alan in 1997 als onderzoeksassistent in het cluster Salmonella en Campylobacter van de afdeling Bacteriologie van het toenmalige ID-DLO te Lelystad, onder begeleiding van Dr. Birgitta Duim en Prof. Jaap Wagenaar.

In de zomer van 2000 startte Alan als AIO in de TSE groep van de afdeling Bacteriologie van het toenmalige ID-Lelystad (voorheen ID-DLO), waar hij onder begeleiding van Dr. Alex Bossers onderzoek deed aan scrapie. De resultaten van dit onderzoek vormde de basis voor dit proefschrift.

Alvorens de mogelijkheid tot promoveren te krijgen heeft Alan van februari 2007 tot en met december 2009 gewerkt als onderzoeksassistent bij het cluster Aviare Influenza van de afdeling Virologie van het toenmalige CIDC-Lelystad (tegenwoordig CVI van Wageningen UR). Vervolgens is Alan als onderzoeksassistent (tot op heden) betrokken bij de afdeling Virologie van het departement Infectieziekten & Immunologie aan de faculteit Diergeneeskunde van de Universiteit van Utrecht.

List of Publications.

Prion related publications.

Prion protein self-interactions: A gateway to novel therapeutic strategies?

Rigter A, Langeveld JP, van Zijderveld FG, Bossers A.

Vaccine. 2010 Nov 18. 28; 7810–7823.

Prion protein self-peptides modulate prion interactions and conversion.

Rigter A, Priem J, Timmers-Parohi D, Langeveld JP, van Zijderveld FG, Bossers A.

BMC Biochem. 2009 Nov 30; 10:29.

Mapping functional prion-prion protein interaction sites using prion protein based peptide-arrays.

Rigter A, Priem J, Timmers-Parohi D, Langeveld JP, Bossers A.

Methods Mol Biol. 2009; 570:257-71.

Mapping of possible prion protein self-interaction domains using peptide arrays.

Rigter A, Langeveld JP, Timmers-Parohi D, Jacobs JG, Moonen PL, Bossers A.

BMC Biochem. 2007 Apr 12; 8:6.

Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion.

Rigter A, Bossers A.

J Gen Virol. 2005 Sep; 86(Pt 9):2627-34.

***In vitro* conversion of normal prion protein into pathologic isoforms.**

Bossers A, Rigter A, de Vries R, Smits MA.

Clin Lab Med. 2003 Mar; 23(1):227-47.

Other publications.

Only two residues are responsible for the dramatic difference in receptor binding between swine and new pandemic H1 hemagglutinin.
de Vries RP, de Vries, E, Moore KS, Rigter A, Rottier PJM, de Haan CAM.
J Biol Chem. 2010 Dec 20. [Epub ahead of print]

A single immunization with soluble recombinant trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1.
Cornelissen LA, de Vries RP, de Boer-Luijtze EA, Rigter A, Rottier PJ, de Haan CA.
PLoS One. 2010 May 14; 5(5):e10645.

Homonucleotide stretches in chromosomal DNA of Campylobacter jejuni display high frequency polymorphism as detected by direct PCR analysis.
Wassenaar TM, Wagenaar JA, Rigter A, Fearnley C, Newell DG, Duim B.
FEMS Microbiol Lett. 2002 Jun 18; 212(1):77-85.

Differentiation of Campylobacter species by AFLP fingerprinting.
Duim B, Vandamme PA, Rigter A, Laevens S, Dijkstra JR, Wagenaar JA.
Microbiology. 2001 Oct; 147(Pt 10):2729-37.

Neonatal sepsis by Campylobacter jejuni: genetically proven transmission from a household puppy.
Wolfs TF, Duim B, Geelen SP, Rigter A, Thomson-Carter F, Fleer A, Wagenaar JA.
Clin Infect Dis. 2001 Mar 1; 32(5):E97-9.

Amplified fragment length polymorphism analysis of Campylobacter jejuni strains isolated from chickens and from patients with gastroenteritis or Guillain-Barré or Miller Fisher syndrome.
Duim B, Ang CW, van Belkum A, Rigter A, van Leeuwen NW, Endtz HP, Wagenaar JA.
Appl Environ Microbiol. 2000 Sep; 66(9):3917-23.

Computer-assisted analysis and epidemiological value of genotyping methods for Campylobacter jejuni and Campylobacter coli.

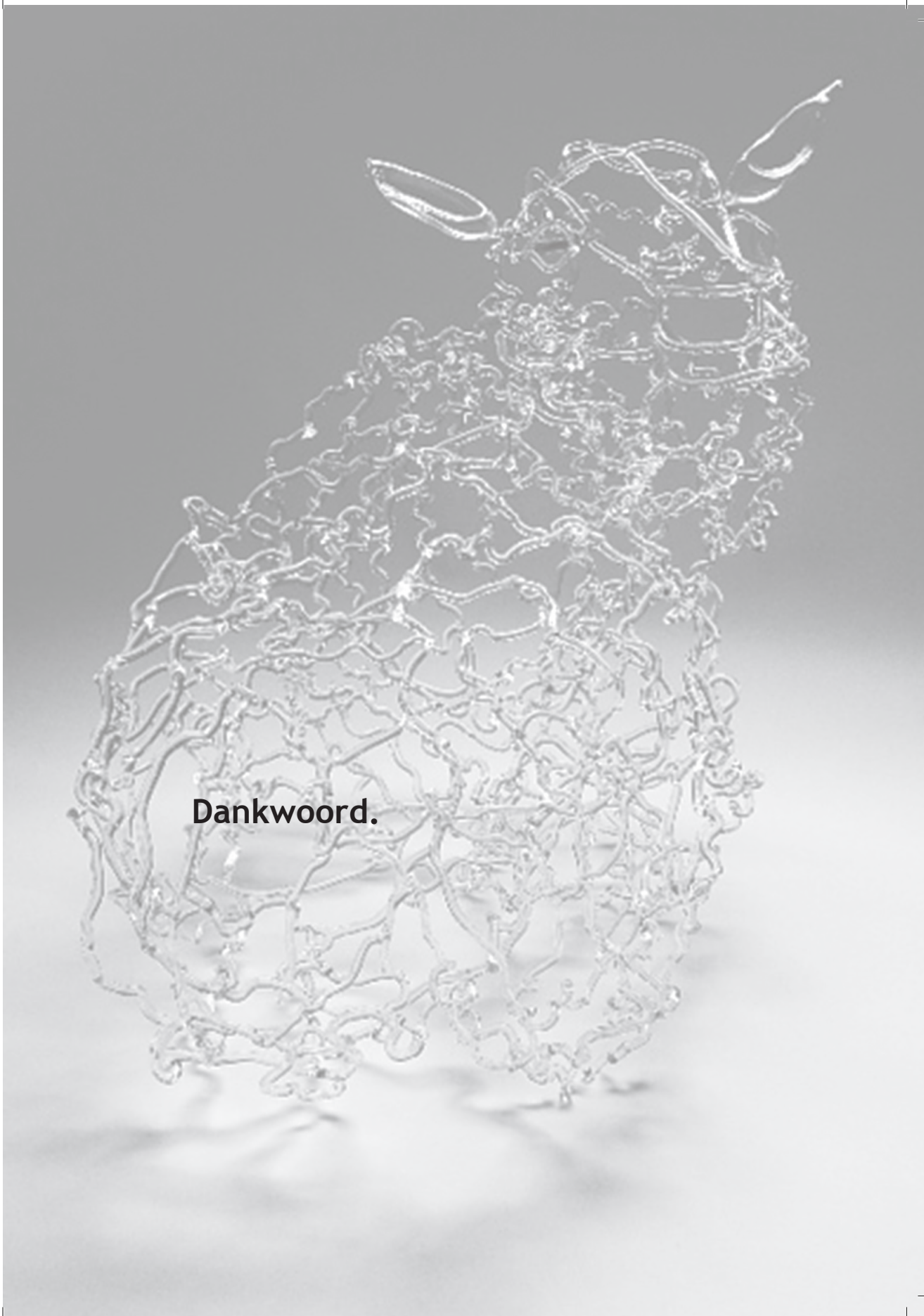
de Boer P, Duim B, Rigter A, van Der Plas J, Jacobs-Reitsma WF, Wagenaar JA.

J Clin Microbiol. 2000 May; 38(5):1940-6.

High-resolution genotyping of Campylobacter strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting.

Duim B, Wassenaar TM, Rigter A, Wagenaar J.

Appl Environ Microbiol. 1999 Jun; 65(6):2369-75.



Dankwoord.

Voor ik hen die belangrijk waren voor het tot stand komen van dit proefschrift ga bedanken wil ik even stilstaan bij de dierbaren die er niet meer zijn om het afronden van de promotie mee te maken. Helaas zijn door ziekte en/of ouderdom de volgende mensen ons ontvallen;

'Tante' Loes Stolker, Hillie Hovenkamp, Oom Bram "beuk" de Jager, (mijn) Opa Deeterink, Ome Joop Henriett, Arrière-grand-mère Georgette Deeterink en Theo Derks.

Aan hen wil ik dit proefschrift opdragen.

"De doorzetter wint!"

Het heeft heel wat voeten in de aarde gehad, maar hier is het dan – mijn proefschrift.

Te beginnen mijn dank aan mijn promotor professor Ab Osterhaus en de Universiteit van Utrecht voor het faciliteren van deze promotie. En natuurlijk mijn dank aan het Centraal Veterinair Instituut (CVI) van Wageningen UR voor het faciliteren van het onderzoek wat wordt gepresenteerd in dit proefschrift, in het bijzonder Fred van Zijderveld en mijn co-promotor Alex Bossers voor hun aandeel in het tot stand komen van deze promotie. Graag wil ik mijn promotor professor Ab Osterhaus en de leden van de beoordelingscommissie, professoren Rob J.M. Moormann, Jaap A. Wagenaar, Peter J.M. Rottier en Andrea Gröne bedanken voor hun positieve beoordeling van het manuscript.

Het pad naar promotie is niet zonder tegenslag geweest, persoonlijk en werkgerelateerd; het aanpassen van het onderzoeksdoel, verlies van dierbaren, afronden van labwerkzaamheden in 'eigen' tijd en het afronden naast een voltijd baan. Maar de kracht om door te zetten had ik mede dankzij de steun en/of hulp van vele vrienden en familie.

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Naast Alex was de inbreng van Jan Langeveld ook van onschatbare waarde. Met behulp van jouw expertise hebben we het maximale uit het peptide-array werk gehaald. Verder was je altijd bereid manuscripten door te nemen en deze met je suggesties te verrijken.

En dan niet te vergeten de fijne samenwerking met Jan Priem en Drophatie Timmers-Parohi. Jullie hebben me (zeker in de eindfase) veel werk uit handen genomen en mooie data aangeleverd.

Ook wil ik graag Prof. dr. J.G. 'meneer' van Bekkum bedanken voor zijn inbreng tijdens de maandelijkse TSE besprekingen. Uw onverminderde interesse en ervaring gaven u een uniek perspectief en inspireerde om vanuit een andere invalshoek naar het onderzoek te kijken.

Een luisterend oor vond ik vaak bij Ruth (Bossers). Zeker toen in een relatief korte periode meerdere dierbare mensen voortijdig wegvielen. Je had het altijd door wanneer ik het moeilijk had en je liet me dan praten, praten en nog eens praten. Ik heb het misschien niet eerder zo gezegd, maar die gesprekken hebben me erg geholpen.

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Als laatste (niet de minste) mijn "carpool-buddy" Peter Willemsen, ongeveer twaalf jaar hebben we heen en weer tussen Amsterdam en Lelystad gereisd, later ook samen met Jantien. In de morgen relaxed, met het nieuws op de radio wakker worden op weg naar werk en 's middags regelmatig in allerlei discussies verwikkeld (of ik dommelend na een lange dag) op weg naar huis. In de auto heeft Peter ook mijn muzikale horizon verbreedt. Van Zappa tot Bulgaars dameskoor tot Lars Holmer en vele andere artiesten. Niet altijd helemaal mijn smaak (toen ik voor het eerst experimenteel werk van Zappa hoorde dacht ik naar de "Jostiband on acid" te luisteren), maar altijd interessant. Natuurlijk was ik als Peter niet kon erg blij met de andere "Amsterdammers" waar ik mee mee kon rijden; Marieke, Herman, Jessica en Arni, bedankt daarvoor.

Ook de collega's aan de "overkant" op de Houtribweg wil ik danken voor alle gezelligheid binnen en buiten de High Containment Unit. Net als op de Edelhertweg waren ook hier teveel collega's om iedereen te bedanken, echter een paar wil ik persoonlijk bedanken. Allereerst Ben Peeters, ik kon bij jou aan de slag aan Aviare Influenza. Niet alleen kreeg ik de kans om mijn werkervaring te verbreden met virologie, maar je gaf me ook de mogelijkheid om de laatste proeven voor het afronden van de promotie uit te voeren / aan

te sturen. En natuurlijk de directe collega's in het moleculaire lab, het was prettig samenwerken en dan niet te vergeten de lolletjes en discussies aan 'onze' tafel in de kantine met de AI groep. Persoonlijk heb ik ook veel gehad aan de werkgerelateerde discussies onder andere tijdens de werkbesprekingen met Jos, Matthijn, Jeroen, Ben, Guus en Riks. Het is altijd prettig om dingen vanuit verschillende perspectieven te bekijken.

During the last couple of years in Lelystad several foreign PhD students started working in Lelystad. A great group of people with whom I enjoyed drinks, dinners, movie-nights. Amongst you I found friendship and a Lowlands-buddy. I hope we stay in touch now I am not in Lelystad anymore and I hope we do so for a long time in the future (it is always great to have people and places to visit abroad).

Tijdens mijn periode in Lelystad heb ik deel uit mogen maken van het Roparun team dat uit collega's van zowel de EHW als de HRW bestond. Het idee was afkomstig van Arie Kant en aangezien het idee achter de Roparun geld inzamelen voor palliatieve zorg bij kanker is, wist ik dat ik hierbij betrokken wilde zijn. De eerste keer als chauffeur en de tweede keer als begeleidend fietser. Graag wil ik iedereen bedanken voor hun inzet voor en tijdens de Roparun. Het was een ervaring die ik niet had willen missen.

Tijdens de laatste loodjes (tot op heden) ben ik werkzaam bij de faculteit Diergeneeskunde, dept. Infectieziekten & Immunologie, afdeling Virologie. Toen ik op zoek was naar een nieuwe baan werkte ik vanuit Lelystad samen met de Influenza groep daar. Erik, jij attendeerde mij erop dat er een positie beschikbaar was. Uiteindelijk leidde dit tot mijn aanstelling en ik dank jullie; Erik, Xander en Peter (prof. Rottier) dan ook voor het in mij geplaatste vertrouwen. Ondertussen werk ik met veel plezier in Utrecht en dat komt zeer zeker mede door alle collega's en studenten.

Dan wil ik ook nog even het woord richten aan de vrienden buiten het lab. De getoonde interesse in mijn werk, de gesprekken (waar ik aan mezelf merkte dat ik nog steeds gedreven over mijn werk praat) bevestigde dat opgeven geen optie was. Met sommigen ging ik ook er even tussenuit naar Oerol, naar Lowlands of een concert (vooral met 'clan' Den Haag). Stappen, borrelen, uit eten, verjaardagen met pindasoep, korte vakanties, kaart-avonden (met de HLO mannen), 'sushi-dates', ceremoniemeester (voor Raymond en Sacha) of

getuige (voor Paul en Anja), alles zorgde ervoor dat ik met beide benen op de grond bleef en tegenslag kon relativeren. Een aantal personen wil ik in het bijzonder bedanken. Raymond, Niels, Laurien, Erik en Jeroen, we waren maatjes op de HLO en ik ben blij dat we nog steeds een goed contact hebben, ik heb het gevoel dat ik altijd bij jullie terecht kan. Sacha, ook hield ik je eerst voor de gek door te zeggen dat ik "verkoper van eetbaar ondergoed op de Albert Kuijp" was, toch mocht ik ceremoniemeester zijn op het huwelijk van Raymond en jou. De van Ekeren-tjes; Paul, je was mijn "intro-kind" en bent één van mijn beste vrienden. Anja & jij verraste me met de vraag om getuige te zijn bij jullie huwelijk, ik was vereerd. Suus, je bent en blijft m'n moppie en ik reken mezelf rijk met zo'n lieve vriendin. Ralph, op de boot naar Oerol hebben we bekokstoofd dat je me ging helpen met de layout van dit proefschrift, dank voor alle hulp & de mooie omslag.

Als laatste wil ik mijn paranimfen bedanken;

Ingrid, zonder wie ik niet mocht proveren. Je bent "de moeder van de kleinkinderen van mijn ouders", m'n "zussie" en ik had het niet zonder jou willen doen. Ruth, je hebt het hart op de juiste plek en ik ben vereerd dat je ook dit voor mij doet.

"Happiness and success in life cannot be measured in possessions or money. It is defined by the love and friendships one is fortunate to accumulate"