Development of llama single-domain antibodies as ingredient for an HIV -1 entry-inhibitor microbicide

De ontwikkeling van lama zware keten antilichamen als component van een microbicide ter voorkoming van HIV-1 infectie.

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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"And those who were seen dancing were thought to be insane

by those who could not hear the music."

F. Nietzsche

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Content

Introduction

HIV-1 BACKGROUND

The Human Immunodeficiency Virus type 1 (HIV-1) is a retrovirus that causes AIDS in humans and probably represents the worst pandemic to have affected mankind in recent history. At the beginning of 2010, despite a decrease of new infections of about one fifth compared to 1997, an estimated 33.3 million adults and children were living with HIV-1, the highest number since the beginning of the pandemic (UNAIDS, 2010).

Identified for the first time in 1983[1], HIV-1 is thought to have evolved from its simian counterpart, SIV, in the early twentieth century when it crossed the species barrier[2]. Even though individuals were infected more than a hundred years ago, it was only in the 1960's when an unknown person carried an easily transmissible variant to Haiti, that the current pandemic likely started[2].

HIV-1 evolved from SIVcpz (chimpanzee), as opposed to HIV-2 that originated from SIVsm (sooty mangabeys). The main differences between the two are the virulence[3] and the genome recombination rate, both higher in HIV-1[4]. Genetically it is possible to identify three groups of HIV-1, M, N and O, deriving from three distinct event of species jump. By far group M is the most widespread, and continues to evolve. At least eleven clades (A to K)[5] and fifteen circulating recombinant forms are known[6] within this group.

Transmission of the virus, which is carried along with bodily fluids, is possible via many routes even though, globally, infections caused by sexual intercourse account for the vast majority of the cases (Figure 1). Other ways of viral transmission are blood transfusions, intravenous drug use by means of a shared needle and childbirth. Since the beginning of the pandemic, mother-to-child transmission and blood products transmission have decreased steadily due to improved health conditions and awareness, especially in developed countries[7][8]. Sexual intercourse on the other hand, remains the main transmission route, accounting for more than 80% of new infections, with a "per-act" infection rate 4 to 10 times higher in low-income countries compared to developed countries.

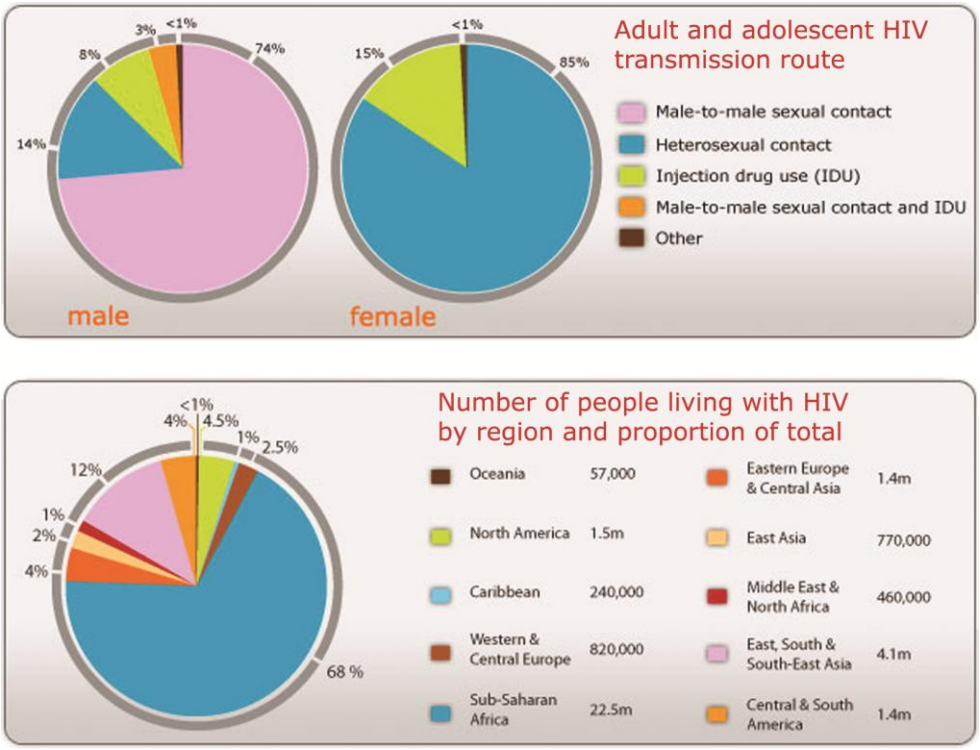

Figure 1. Top panel: HIV transmission routes among adults and adolescents in the US in 2009, by gender. Bottom panel: distribution of people living with HIV worldwide, by region[9].

The course of infection can be divided in three phases: acute (primary infection), chronic (clinical latency) and terminal, the latter leading to immune collapse and development of AIDS (Figure 2).

In the acute phase primary target cells (CD4 positive T cells) are infected, preferentially those lacking activation markers and expressing low levels of chemokine receptor CCR5[10]. Rapid expansion of HIV-1 takes place in the gut-associated lymphoid tissue (GALT) first and then systemically[11]. Along with a sharp rise in plasma levels of viral RNA (up to 10^6 copies per ml of plasma), irreversible destruction of reservoirs of helper T cells is observed, which is clinically important because in the past it has dwarfed early treatment

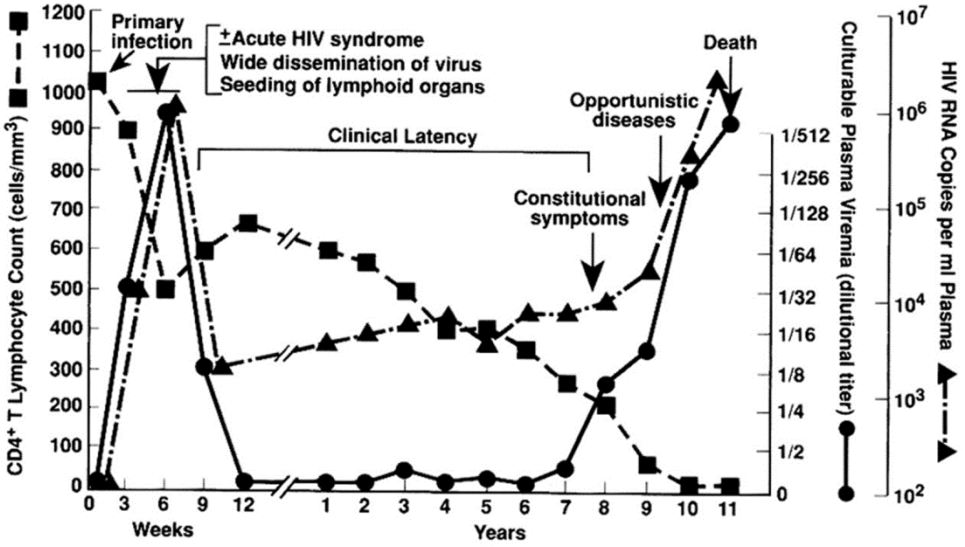

Figure 2. The complex, multifactorial, multiphasic, and overlapping factors of the immunopathogenic mechanisms of HIV disease are shown. Throughout the course of HIV infection, virus replicates and immunodeficiency progresses steadily, despite the absence of observed disease during the so-called clinical latency period. Immune activation and cytokine secretion vary among HIV-infected persons, sometimes increasing dramatically as disease progresses. Immune activation and cytokine secretion play a major role in pathogenesis[13].

approaches and establishes viral latency. Within six to twelve weeks the viremia sets to a lower titer, a "set point", which is predictive of the rate of progression to AIDS[12]. The number of circulating and mucosal CD4 positive T cells is partially re-established and is destined to slowly decrease during clinical latency. When it will reach the threshold of 200 units/mm^3 it will be the beginning of the terminal AIDS phase. The chronic phase duration may differ between individuals, up to decades, depending on many factors, including the time of antiretroviral therapy initiation and host genetic and immune factors. Among seropositive patients, a small group of individuals has been identified that can control viral load even in absence of antiretroviral therapy. They are called long-term nonprogressors, or elite controllers, and understanding what factors determine this status could lead to development of vaccine or more efficient drugs.

HIV-1 STRUCTURE AND ENTRY MECHANISM

HIV-1 is an enveloped lentivirus member of the Retroviridae family. Its genetic information is carried by a single–stranded RNA molecule present in two copies and tightly bound to nucleocapsid protein p7, encased in a conical capsid composed of protein p24. Reverse transcriptase, integrase, protease and ribonuclease enzymes are also present within the capsid, to help the integration of genetic information in the host genome. Matrix protein p17 surrounds the capsid and underlies the viral envelope, which consists of the double-layer phospholipid membrane of the host cell the virus originally budded from. The only viral protein present on the surface is Env, the mushroom-shaped spike that allows the virus to attach to and infect target cells, and the only antigenic determinant that the immune system raises antibodies against[14] (Figure 3).

Structure of HIV-1 Env

Because of the utmost importance of Env in the infection mechanism, its structure has been extensively studied. At genome level, Env is encoded by one single gene but after translation the protein precursor gp160 is proteolytically cleaved into two proteins gp120 and gp41[16, 17]. Three gp120-gp41dimers together form the heterotrimer.

Gp120 consists of a heavily glycosylated, double β-barrel outer domain, an inner domain that includes a 3-helix, 4-strand bundle, a 7-stranded β-sandwich and a 4-strand bridging sheet composed of two strands from the inner domain and two strands from the outer domain (Figure 4). This structure does not resemble any other known protein structure[18]. The determination of its structure has not been an easy task, because of the high degree of glycosylation, accounting for about 40-50% of the molecular weight[19, 20] and the need to stabilize the conformation by adding ligands that allow the formation of well-ordered crystals or by deleting the variable regions[21]. It follows that the available crystal structures of gp120 represent specific conformations that the protein assumes when bound to CD4 or gp120-specific antibodies.

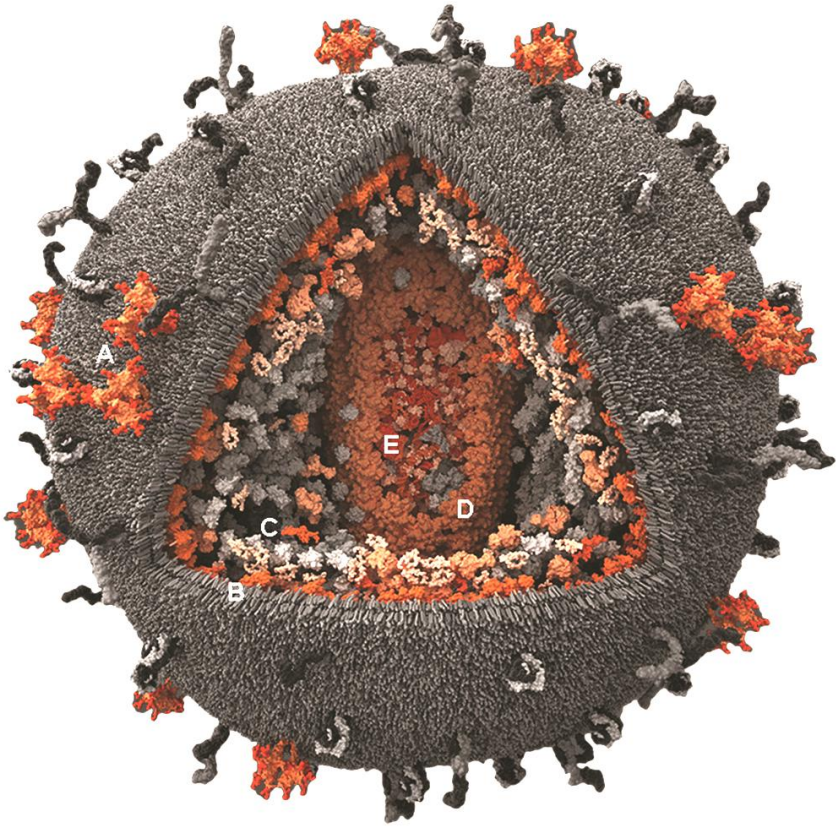

Figure 3. Computer-generated model of HIV virions. From the surface to the core of a virion, different structural elements are highlighted in shades of red. A) The viral surface proteins gp41/gp120 are encoded by the Env gene and are synthesized as a single precursor protein gp160, which is glycosylated by asparagine-linked high-mannose oligosaccharides and processed by host protease to form two non-covalently associated subunits. After processing, gp41 subunit remains a transmembrane protein, while the gp120 subunit is non-covalently bound to gp41 and interacts with CD4 receptor of the host cell. B) Directly below the viral lipid membrane there is a matrix layer composed of p17 protein which is the product of viral Gag gene. In mature virion, p17 forms trimers. Each subunit of the trimer is myristoylated and anchored in the membrane. C) P6, a small protein within the polyprotein encoded by the gag gene, is found between matrix and capsid. It is important for virion budding and for incorporation of virus-encoded protein Vpr into the particle. Vpr performs several crucial functions in HIV life cycle, including nuclear import of viral DNA. D) HIV capsid is a cone-shaped structure composed of approximately 250 hexamers and 12 pentamers of p24 protein, encoded by the Gag gene. E) HIV capsid contains viral RNA and enzymes, reverse transcriptase and integrase, needed for copying viral RNA into DNA and inserting it into the host genome. HIV genetic material is presented by two copies of single-stranded positive-sense RNA molecules, each approximately 10000 nucleotides long. RNA is tightly bound by p7 proteins, also encoded by Gag (HIV model and caption by www.visualscience.ru)[15].

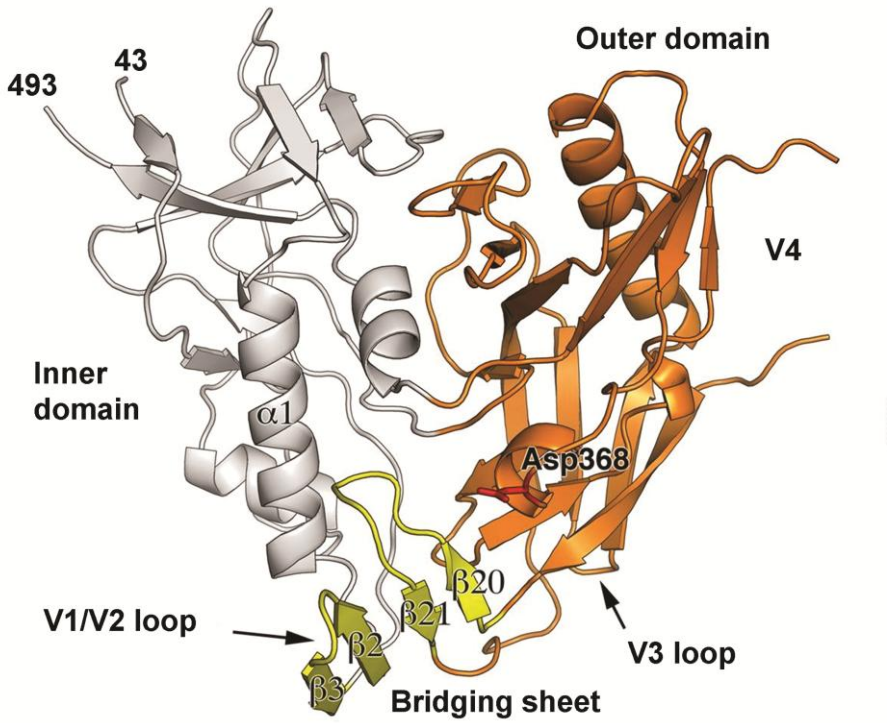

Figure 4. Crystal structure of deglycosylated gp120 core monomer with complete inner domain in complex with CD4(D1D2) and Fab 48d (ligands not shown.The inner domain (grey), outer domain (orange), and bridging sheet (yellow), composed of the V1/V2 stem and β20-β21 hairpin, are the main structural features found in the CD4-liganded conformation. The V1/V2 and V3 loops are truncated. Asp368, which interacts with Arg59 from CD4, is depicted as red sticks (Figure and caption by Pejchal R, Wilson IA, Current Pharmaceutical Design, 2010)[18].

Each gp120 unit is noncovalently associated with the membrane-spanning gp41. Gp41 is composed of a transmembrane region (TM), a membrane proximal external region (MPER), a C-terminal and a N-terminal heptad repeat region (CHR and NHR respectively) that are connected by a loop stabilized by a cysteine bridge, and a N-terminal fusion peptide (FP)[18]. The relative conformation of CHR and NHR is very important for the membrane fusion process, as described later. Unlike gp120, gp41 shares high sequence

homology with other viruses, and the post-fusion structures of their fusion proteins are also similar[22-24]. Nevertheless, because of the large, fusion-driven, conformational rearrangements that are virus-specific, little information can be obtained by looking at other pre-fusion structures; therefore a complete, pre-fusion gp120/gp41 trimer would be suitable.

Despite a gp120/gp41 trimer crystal structure is still missing, cryo-electron tomography has provided a reconstruction of the full HIV-1 Env based on the BaL isolate[25] that is fitting with the existing partial crystal structures. The reconstruction was based on gp120 crystallized with soluble CD4 molecules (sCD4) as well as specific antibodies. It showed that various conformational states are present, from closed, to intermediate (immunoglobulin b12-bound state), to open (sCD4 and immunoglobulin 17b-bound state). It emerged that in the closed state the gp120 monomers are rather separated, with a reduction in density at the center of the spike. In the intermediate state, gp120 monomers appear to expand to accommodate the binding of b12 and in the open state a significant rearrangement and rotation of the gp120 units occurs accompanied by a likely loss of gp120-gp120 interactions. The V1/V2 regions of the inner domain are not clearly localized in the close and intermediate states, but it is certain that they are involved in the formation of the bridging sheet in the open (CD4-bound) state, together with β20- β21 strands of the outer domain. Some models also considered the glycans that abundantly decorate Env and observed that the only significantly large exposed region is the CD4 binding site[26] (Figure 5).

Infection mechanism

To infect a cell, HIV-1 must transfer its RNA across both viral and cellular membranes, not an easy task, considering the stability and evolutionary role of biological membranes -to contain and protect from external attacks. The entry process can be divided in three steps: 1) the recognition and binding to cellular receptor CD4, which triggers a conformational change of gp120, 2) the binding to a coreceptor cell-surface protein by the now available coreceptor-binding site and 3) the membranes fusion reaction itself, mediated by gp41.

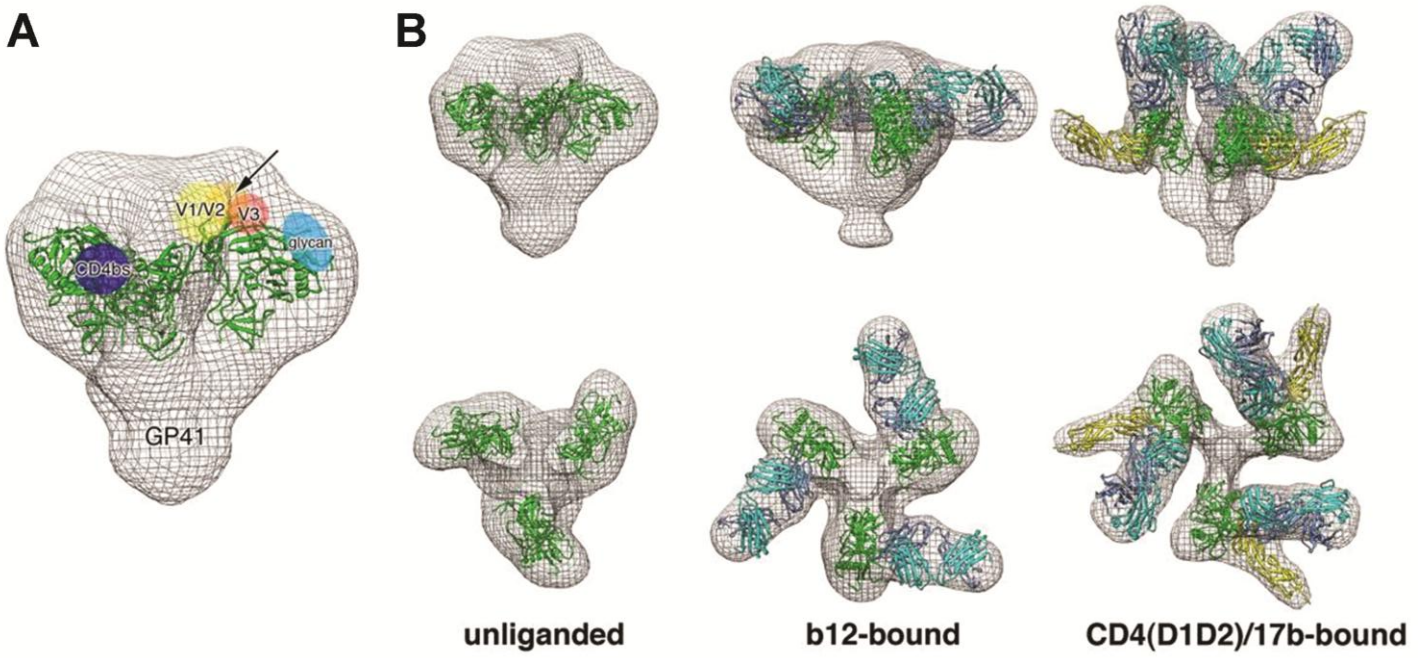

Figure 5. Trimer cryoelectron tomographic reconstruction density. A) Side-view of cryo-ET density for an unliganded BaL trimer that is fitted with the crystal structure of a b12-bound gp120 core monomer. B) Side- and top-views of unliganded trimer density fitted with the b12-bound gp120 core monomer (left), b12-bound trimer density fitted with b12-bound gp120 monomer in complex with b12 Fab (middle), CD4/17b-bound trimer density fitted withCD4/17b-bound gp120 core monomer in complex with CD4(D1D2) and 17b Fab. These three reconstructions represent the closed, intermediate and open states of the trimer. Coloring scheme: gp120: green, Fab: cyan (light chain) and dark blue (heavy chain), and CD4 (yellow). The arrow indicates the approximate localization of V1/V2 regions (Figure and caption by Pejchal R, Wilson IA, Current Pharmaceutical Design, 2010)[18].

Receptor binding

Env consists of two glycoprotein trimers, three units of the transmembrane gp41 and three of gp120, which are noncovalently associated. The first interaction with target cells occurs between gp120 and the cell receptor CD4, explaining the propensity of HIV-1 to infect cells of the immune system and eventually leading to immune dysfunction. The CD4 binding site on gp120 is located in a cavity between the inner and the outer domain and the bridging sheet. Of the four extracellular domains of CD4, those involved in the binding are the farthest ones from cell surface, D1 and D2. Although CD4 binding is a requisite, other molecules such as the cell surface receptor DC-SIGN present on dendritic cells, especially Langerhans cells present in the mucosal layer, can capture the virus and shuttle it in an infective form to areas where more primary target cells are concentrated[27, 28]. This type of interaction may become very important when developing drugs that prevent virus entry (entry inhibitors).

Co-receptor binding

Upon binding to CD4, gp120 undergoes a conformational rearrangement. The formation of a depression called "Phe43 cavity" and the stabilization of the two strands (V1/V2 and β20-β21) that constitute the bridging sheet are evidence for the large change in conformation[29]. Most interesting is probably the disclosure of the V3 loop that becomes exposed and can subsequently bind to an appropriate coreceptor[30], such as the CCR5 or CXCR4 chemokine receptors[31]. The coreceptor choice, which determines the tropism of the virus (i.e. R5 or X4) is preeminently dependant on variable regions in gp120 V3, and to a lesser extent, on V1/V2[32, 33]. A highly conserved region involved in coreceptor binding that lies between V1/V2 and V3 is revealed in the CD4-bound conformation and has been suggested to be a promising target for antiviral drugs[34]. Interaction with the acidic N-terminal portion of the coreceptor is facilitated by the basic amino acids on the surface of CCR5 binding site[35, 36].

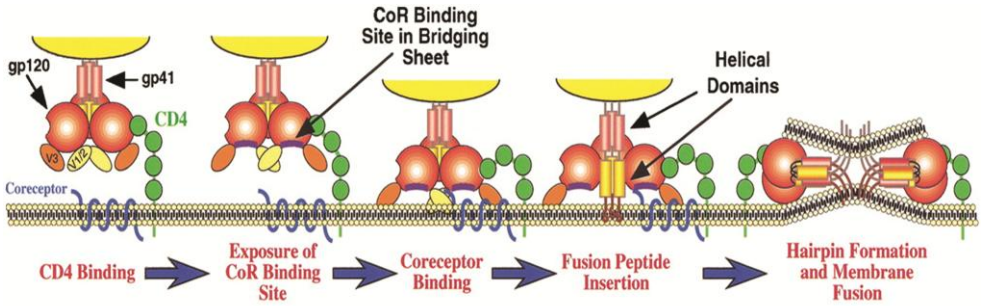

Figure 6. Model for HIV-1 infection mechanism. Binding of CD4 to gp120 results in exposure of a conserved coreceptor (CoR) binding site in gp120, perhaps by movement of the V3 and V1/2 loops. Coreceptor binding causes the fusion peptide of gp41 to be exposed and inserted into the membrane of the target cell in a triple-stranded coiled-coil. Formation of a helical hairpin structure in which gp41 folds back on itself is coincident with membrane fusion (Figure and caption by Doms RW, Trono D, Genes & Development, 2000)[43].

Membrane fusion

It is not yet clear how the information of the bound coreceptor on gp120 is passed to gp41, but models exist describing the structural changes on gp41. The N-terminal amino acids of FP are inserted into the cell membrane as the gp41 trimer assumes a conformation called extended coiled-coil, where the six α-helices of CHR and NHR are exposed and form a coil. The hydrophobic grooves (leucine/isoleucine repeats) generated by the coils at the N-terminal region serve as a base into which the CHR will pack upon formation of a structure named six-helix bundle or hairpin[37, 38]. The hairpin is a stable structure that brings the viral and cell membrane in contact and therefore it likely represents the final conformation of gp41, rather than a fusion intermediate[39]. The change in free energy measured on its formation was shown to be sufficient to the formation of a fusion pore[40]. Necessary for membrane fusion is the cooperation of several HIV-1 spikes. It is currently estimated that multiple CD4 molecules[41], four to six CCR5 and three to six Env are needed for a successful fusion event [42]. This necessary cooperativity of several (viral and cellular) factors could be exploited by multi-target entry inhibitor drugs that would not need to block all the possible epitopes to prevent membrane fusion (Figure 6).

MICROBICIDES

Therapeutic strategies have been developed since the mid nineties, with the discovery of drugs with antiretroviral activity (ARV). Antiretroviral therapy (ART) has since then greatly improved life expectancy of people carrying the virus. In the early nineties, before the ART era, it would take approximately 15 months from seroconversion to developing AIDS, whereas it is nowadays estimated that a person will live about 20 years with very low viral load, before AIDS symptoms will appear. This is due to the improvement that antiretroviral drugs have undergone since they were first introduced. A wide range of molecules are now available that target different steps of the virus replication cycle. Reverse transcriptase inhibitors (Zidovudine, Lamivudine, Didanosine), Integrase inhibitors (Raltegravir), Protease inhibitors (Indinavir, Saquinavir), Entry inhibitors (Maraviroc, Enfuvirtide) have demonstrated that their combined use may slow down disease progression, reverse -at least temporarily- the symptoms of the late stage of the disease, and reduce the infectivity of the patient so that he/she is less likely to transmit the virus to the partner upon sexual contact. ART has shown its efficacy in lowering the mother-to-child transmission rate at birth, too.

Despite the above, drug therapy has also important drawbacks. ARV drugs are so expensive that not only cause a significant economic burden to the healthcare system of affluent countries, but many resource-limited countries where the epidemics rages can simply not afford to provide treatments to those who need it. Even though remarkable improvements have been made in formulations and dosage, harsh side-effects are common to seropositive people, who are bound to a drug regimen for the rest of their life. Moreover in the long term, emergence of drug-resistant strains is likely and more drugs are needed to delay the onset of AIDS symptoms.

As long as a prophylactic vaccine will remain elusive, prevention is the best way to avoid HIV-1 transmission. Both a behavioral approach and a biology-based intervention are desirable. If the first one is more pertinent when discussing of socio-economic matters, the latter is addressed in this work. A biology-based preventative approach involves the topical application of

compounds that prevent the very early stage of sexual transmission, namely the infection of target cells in the exposed host.

In the scenario of a limited-resource country, the most important rationale for developing a microbicide is to give a woman the possibility to protect herself. In sub-Saharan Africa the HIV prevalence among young, sexually active women is three times higher than among men of the same age group[44]. The majority of infections are acquired through sexual intercourse. Because of social reasons women might not be in the conditions of negotiating the use of condom –the most efficient way of preventing infection, together with abstinence - therefore they need protection in a form that they themselves can control. A discrete, self-applicable, topical microbicide would be tolerated (or not even noticed) by the male partner and would be acceptable in societies where fertility has a high value[45].

Requirements for a successful microbicide

It was stated that a useful microbicide must safe, acceptable, efficacious and affordable[46]. It should not cause chronic inflammation, or worse be carcinogenic when applied regularly for extended periods. The pH and vaginal flora should be maintained because they are a natural defense against the virus[47]. Compounds of proteinaceous nature should be checked for immunogenicity, moreover they should not affect the activation state of local immune system cells or their concentration close to areas where the virus could infect them more easily[48].

Acceptability, for both partners, is also an important factor. Formulation studies should look into the smell, the viscosity and the color of the compound (gel, film or cream). It is not a given that every active molecule can be formulated in any possible way. The formulation also defines the intervals at which the microbicide should be applied. A coitus independent application should be sought after, as it's possible that the conditions before the intercourse do not allow proper application. A once-daily application might become the routine and therefore adherence would increase, even though formulating the drug as e.g. an intravaginal device to be put in place only once

a month might prove even more efficacious. Menstrual cycle should be taken in consideration too.

The economic aspect is probably the most relevant in the contest of sub-Saharan countries. If the manufacturing cost is too high it will be unaffordable by countries that most need it. Several studies have speculated on what the cost-per-dose, and in this respect a once-per-month formulation will help reducing the financial burden. Moreover some molecules with very promising activity could be too complicated to produce or their scale-up not possible.

Many, if not all these aspects have been considered in the design of the study reported in chapter four. There we have thought what challenges a protein-based microbicide will have to face and we systematically analyzed them in order to evaluate whether small molecules like llama antibody fragments have the potential to become a useful drug.

LLAMA HEAVY-CHAIN ANTIBODY FRAGMENTS

Structure and properties

The serum of llamas and other members of the camelidae family is known to contain a considerable fraction of heavy-chain antibodies that lack the light chain typical of immunoglobulins[49]. Their heavy chain consists of only three globular domains, of which the second and third (CH2-CH3) are highly homologous to the Fc domains of conventional antibodies[50, 51], whereas the CH1 is missing and replaced by a hinge. Therefore the antigen-binding domain is reduced to a single variable domain (VH), referred to as VHH that is adapted to be functional in the absence of a light chain[52, 53] (Figure 7). It consists of a 15 Kd globular protein with the typical Ig fold composed of two β -sheets, one of four and one of five β-strands connected by loops at the bottom and at the top of the structure[54]. Three of these loops are hypervariable regions called complementarity determining regions (CDR) and are primarily involved in antigen binding. The homology to VH domains of conventional antibodies is obvious, but there are a few differences. CDR1 and CDR3 are usually longer[55], and the N-terminal part of CDR1 is more variable[56, 57]. The amino acids occurring in framework 2 (i.e. between CDR1 and CDR2) are more hydrophilic in VHH compared to VH as a consequence of the evolution of heavy-chain antibodies that lack the VL domain[58]. Crystal structures of VHHs revealed that CDR loops assume conformations not seen in conventional Igs[59, 60] and this, together with the extended length of their CDR1 and 3 might compensate for the lack of the three CDRs carried by VL and increase the surface of the VHH paratope. Interestingly, the shape of the paratope based on only three CDR loops is more often convex, as opposed to the flat or concave shape of conventional paratopes. This property, together with a finger-shaped CDR3[61] allows VHH to penetrate deep into clefts or pockets that are normally unreachable by the larger conventional antibodies[62].

Due to their single-domain nature and higher hydrophilicity of the residues conventionally located at the VH-VL interface, VHH display interesting properties of high thermal stability[63, 64], refolding capacity after denaturation[65] and good tissue penetration[66, 67].

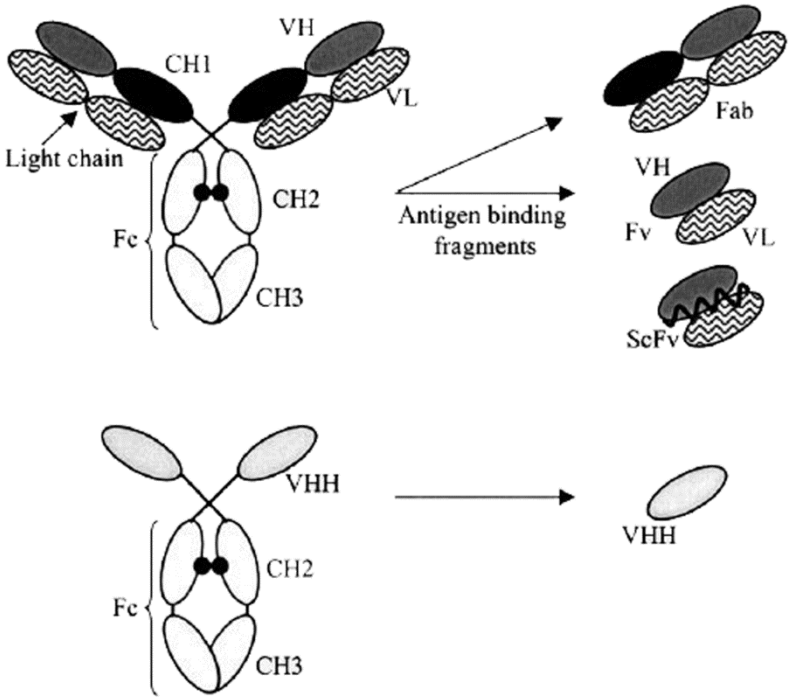

Figure 7. Schematic illustration of the conventional (top) and heavy-chain IgG antibodies (bottom) present in llama serum. The entire light chain (curved lines) and CH1 domain (black) are absent in heavy-chain antibodies. The antigen-binding domains of conventional antibodies obtained after proteolysis (Fab) or after cloning, and expression of the gene VH and VL fragments are shown. A synthetic linker introduced between the VH and VL stabilizes the VH–VL dimer and forms the scFv. The recombinant VHH, the variable domain of heavy-chain antibodies is obtained after cloning and expression. The VHH is the minimal intact antigen-binding fragment that can be generated[68].

Selections

Isolation of mRNA from blood, lymph nodes or spleen lymphocytes of (non) immunized llamas, conversion of mRNA into cDNA and PCR-cloning of the V domain of heavy-chain immunoglobulin reservoir generate the VHH pool which is then inserted into a phage-display plasmid[58, 69]. Phagemid libraries can be constructed, containing up to 10^8 VHH clones[70] displayed as fusion protein on the tip of gIIIp of the phage. With a few rounds

of panning or capturing it is possible to isolate single phages containing VHH genes with specificity for virtually any antigen. This method for selecting VHH is simpler and more efficient than corresponding methods based on scFv or Fab libraries that require the combination of two genes (VH and VL) for the assembly of the functional antibody fragment.

VHH selected from immune libraries have proved very high specificity for their cognate antigen[71], with affinities in the low nanomolar range[7273]. Improved outputs can be obtained when selections are made under conditions of increased stringency (higher temperature, high or low salt concentration, high or low pH, and low antigen concentrations)[74]. Specific conditions can also be applied during selections, in order to obtain VHH resistant to proteolytical degradation or denaturing agents.

Applications

There is growing interest in developing VHH for a large range of applications. VHH were isolated for use in anti dandruff shampoo[75], in biosensors[76], as imaging tools[77] or as reagents in affinity purification[78]. They have great potential in medicine as diagnostic, preventative and therapeutic molecules as well.

VHH were described with neutralizing properties against Rotavirus-caused diarrhea in humans. Despite passing through the acidic environment of the stomach, VHH were still active and induced protection of the host most likely by masking crucial epitopes on the virus spike[79]. Bacterial diarrhea, caused by *E. coli,* was also inhibited efficiently *in vitro* by VHH directed to the F4 fimbriae, but they poorly protected piglets *in vivo*, probably because of proteolytic degradation occurring in the gastro intestinal tract[80].

At 15 Kd, the size of VHH monomer allows a rather quick clearance off the blood circulation at renal level and this might be a factor limiting the efficacy of the molecule. In the attempt to extend the half-life, VHH have been targeted to proteins with long residence time in the serum, like albumin[81] or immunoglobulin[82]. Formatting the VHH as heterodimers (one monomer with therapeutic effect and one targeting serum proteins) resulted in great extension

of the half-life to levels similar to the serum protein that is targeted by the "helper" VHH.

VHH binding to the epidermal growth factor receptor (EGFR) were shown to prevent the binding of the EGF to its receptor and therefore efficiently inhibit the growth of solid tumors, especially when formatted as trimer, with two monomers targeting different epitopes on the EGFR fused with one albumin-binding monomer[81]. Hultberg *et al.* reported a synergistic neutralizing effect of up to 4000 times when using multimeric VHH targeting distinct epitopes on the spike protein of the Respiratory Syncytial virus (RSV). Smaller, but very remarkable, improvements were achieved with multimeric constructs specific for the envelope glycoprotein of the Rabies virus and for the hemagglutinin protein of the H5N1 Influenza virus[72].

Most relevant for the purpose of this thesis was the report about selection and characterization of VHH with the ability of potently neutralizing several HIV-1 clades[83] from llamas immunized with the glycoprotein gp120 of HIV-1 primary isolate CN54. In the context of HIV-1 neutralization, a few other studies have also described the successful isolation of antibody fragments directed to viral targets as well as to cellular targets. Anti gp41 VHH were selected, that blocked the conformation of gp41 in a state unable to mediate membrane fusion (L. Rutten, personal communication). Strong antiretroviral activity was also found in VHH directed against the cell surface protein CXCR4, used by HIV-1 as coreceptor for viral entry. This study demonstrated once more the efficacy of linking two antibody fragments recognizing distinct epitopes to reach much higher binding affinity than the monovalent counterparts[84].

Production

The expression of recombinant VHH has been successfully demonstrated in several microorganisms, both prokaryotic and eukaryotic. Their production yield is on average higher than full-size antibodies, but great variation has been recorded between different clones[85, 86]. Chapter three of this thesis deals with sequence-related factors influencing the secretion yield in *S. cerevisiae*.

Bacterial systems, preeminently *E. coli,* are preferred for the ease of cloning VHH constructs, the general good yield of product, and the short development time from gene to product[71, 87]. VHH can be directed to the oxidizing environment of the periplasmic space, or to the reducing cytosol. The latter often requires the extra step of refolding the protein during purification. Production of VHH in *E. coli* implies the presence of the endotoxin lipopolysaccharide (LPS). This makes it non-suitable for therapeutic applications, unless expensive purification steps are undertaken to get rid of LPS[88].

The filamentous fungus *A. awamori* was used to express a VHH fused to a peroxidase enzyme and good yields were obtained even though considerable amount of fusion protein was detected intracellulary[89]. *P. pastoris* is often a favored production host because of extremely efficient secretion, but only recently one report described its use for VHH expression[90]. Much more common is the use of the yeast *S. cerevisiae* for the industrial production of antibody fragments, in particular when they are meant for therapeutic applications or consumer goods[8591 92]. Large scale, high density fed-batch fermentations of *S. cerevisiae* have been in use for several years because of the GRAS (generally recognized as safe) status of this organism, the low production costs and the relative purity of the VHH, secreted in the extracellular medium. Because of the high costs connected with production of conventional antibodies in mammalian cells (Roche has invested several hundred million Euros for new production facilities in Germany and Switzerland (www.roche.com)), less expensive production of VHH becomes very attractive when these molecules are a viable alternative to the use of conventional antibodies.

A number of studies have highlighted factors that influence the secretion level of VHH. Beneficial to secretion in yeast is the presence of N-linked glycosylation sites[93], even though this was shown to have the potential of reducing the specific activity of the antibody fragment[82], or induce immunogenicity in humans because of the yeast-specific oligosaccharides added to the protein[94]. Adding a C-terminal Cys, for inducing dimerisation, had a detrimental effect on the secretion of shark antibody fragments,

structurally similar to VHH[95]. It was also shown that replacing the hydrophobic residues involved in CH1 interactions when expressing scFv had a favorable effect in *E. coli*[96]. Finally, Jespers *et al.* suggested that a correlation exists between high refolding capability and high secretion level of VH domains in *E. coli*[97].

Besides sequence-related factors, culturing conditions might also affect production yield of VHH. *S. cerevisiae* optimally secretes VHH when grown in N-limiting medium, at a specific, slow, growth rate[98]. Using ethanol as sole carbon source in fed-batch fermentations was shown to increase fivefold the productivity of yeast, independently of the protein expressed[86]. *P. pastoris* production could be improved as well by supplementing growth medium with Casamino acids, sorbitol and ethylenediamine tetraacetic acid[90].

Many of the findings described in this section were applied to our fermentation system for the production of anti HIV-1 VHH. Next to the already reported observations, we identified a new set of factors that further optimize the secretion level of antibody fragments in *S. cerevisiae*. Eventually, a systematic improvement of VHH sequences and yeast growth conditions will likely remove the production bottleneck that often curbs the potential of a promising molecule.

AIM OF THE THESIS

The objective of this thesis is to investigate the mechanisms that underlie the ability of llama antibody fragments to neutralize HIV-1 infections by inhibiting the entry of the virus in the target cell. Moreover we examined whether VHH have what it takes to become a microbicidal drug that can be used in limited-resources settings to reduce the impact of sexual transmission of the virus. In wait for a vaccine that will provide long term immunity, prevention seems today the way forward to contain the AIDS pandemic.

Definition of the structure of a HIV neutralizing VHH

In chapter 1 we have crystallized the HIV-1 neutralizing VHH D7 and observed the molecular details of its interaction with gp120 IIIB. The epitope of D7 partially overlaps with that of human mAb b12 on the CD4bs. By means of site-directed mutations and surface plasmon resonance experiments we have defined a few amino acids in CDR3 as the key determinants of the interaction and therefore responsible for the neutralization potency. Mutations that impair the affinity for gp120 affected the neutralization in a proportional fashion. Comparing the structure of CDRs of D7 to those of other anti-CD4bs antibodies we concluded that different modes of interaction exist, most likely attributable to the structural plasticity of the CD4 binding site.

Unraveling the determinants for potent cross-clade neutralization

The second chapter of the thesis deals with the definition of determinants for cross-clade neutralization by VHH A12 and D7. Using a PCR method we constructed libraries of VHH with very high diversity, but still belonging to the same family. Selections on these libraries yielded VHH with amino acid sequence very close to that of parental VHH but with specific binding and neutralization phenotypes. By combining sequence data and binding/neutralization data we could identify in the last three amino acids of CDR3 the crucial interactions defining the specificity of each VHH for a certain HIV-1 subtype.

Studying the folding of VHH to optimize the production efficiency

In the perspective of using broadly neutralizing VHH in microbicides, large scale fermentations will be needed to produce enough material. A key issue is how efficiently VHH are produced. In this chapter we studied the folding of VHH using computational and experimental methods to identify factors that determine improved production yield. Mutagenesis of model VHH confirmed that a few key residues dictate the folding rate and the efficiency of ER escape. We also looked at correlates between the use of specific V or J segments and production yield, and we observed that some J segments are associated with high or low secretion efficiency. We were able to reclone these segments in unrelated VHH and transfer the beneficial or detrimental effect.

The development of VHH in applicable microbicidal drugs

The final chapter of the thesis took a close look at the requirements that were said an applicable microbicide should have to be employed in developing countries. We systematically subjected the neutralizing VHH in our hands with the challenges they will have to face as topical microbicides in sub Saharan Africa. We then showed that they are able to withstand continued and prolonged exposure to high temperatures and acidic environment without decrease in activity, that they are still active after formulation and release from IVR and HEC gel and that when they are applied in the vaginal lumen they would be able to penetrate the mucosal layer and localize where primary target cells are present, in order to carry out their inhibiting activity.

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Chapter 1

Adapted from:

Crystal Structure of the Neutralizing Llama VHH D7 and Its Mode of HIV-1 gp120 Interaction

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HIV-1 entry into host cells is mediated by the sequential binding of the envelope glycoprotein gp120 to CD4 and a chemokine receptor. Antibodies binding to epitopes overlapping the CD4-binding site on gp120 are potent inhibitors of HIV entry, such as the llama heavy chain antibody fragment VHH D7, which has cross-clade neutralizing properties and competes with CD4 and mAb b12 for high affinity binding to gp120. We report the crystal structure of the D7 VHH at 1.5 A° resolution, which reveals the molecular details of the complementarity determining regions (CDR) and substantial flexibility of CDR3 that could facilitate an induced fit interaction with gp120. Structural comparison of CDRs from other CD4 binding site antibodies suggests diverse modes of interaction. Mutational analysis identified CDR3 as a key component of gp120 interaction as determined by surface plasmon resonance. A decrease in affinity is directly coupled to the neutralization efficiency since mutations that decrease gp120 interaction increase the IC50 required for HIV-1 IIIB neutralization. Thus the structural study identifies the long CDR3 of D7 as the key determinant of interaction and HIV-1 neutralization. Furthermore, our data confirm that the structural plasticity of gp120 can accommodate multiple modes of antibody binding within the CD4 binding site.

INTRODUCTION

The envelope glycoprotein (Env) from the human immunodeficiency virus type 1 (HIV-1) forms a heterotrimer composed of the receptor binding subunit gp120 and the membrane anchored fusion protein subunit gp41. Entry into host cells is mediated by gp120 interaction with CD4 that triggers a conformational change allowing subsequent interaction with cellular coreceptors such as CCR5 or CXCR4 [1–4]. Together these events trigger a refolding of gp41 that leads to the fusion of virus and host cell membranes [5–7]. Env is the target for entry inhibitors [8] and neutralizing antibodies directed against gp120 and gp41 [9]. A main problem in HIV-1 vaccine research is the generation of cross-subtype neutralizing antibodies, which is due to the fact that HIV-1 employs a number of strategies to evade the immune response. This includes highly variable gp120 regions, a carbohydrate shield [10] and conformational masking of the receptor binding site [11]. The overall conformational flexibility of gp120 is highlighted by the differences between the native SIV gp120 core structure [12] and structures representing the CD4- and antibody-induced conformations of the HIV-1 gp120 [13–16]. Gp120 structures are composed of an inner and an outer domain; the inner domain varies substantially including the refolding of the bridging sheet, while the outer domain harbouring the CD4 binding site is mostly conserved except for the refolding of the CD4-binding loop [12,13]. The conformational flexibility is considered to be the main obstacle to the development of an HIV-1 vaccine, besides the sequence variability and the glycan shield. Consequently, only few broadly neutralizing antibodies have been described to date [17]. MAbs 2F5, 4E10 and Z13 recognize epitopes within the membrane proximal region of gp41 [18–21], mAb 2G12 recognizes a carbohydrate motif [22,23], b12 interacts within the CD4 binding site [24,25], HJ16 overlaps with the CD4 binding site [26] and antibodies PG9 and PG16 are specific for the trimeric Env conformation [27].

The crystal structure of gp120 in complex with b12 revealed the molecular details including a substantial conserved gp120 surface overlapping between

both the CD4- and b12-bound states [14]. The similarities of both interactions is highlighted by the fact that b12 employs Tyr^{53} to fill the hydrophobic pocket in gp120 that is otherwise occupied by CD4 Phe^{43} [14]. MAb b12 is broadly neutralizing since it engages gp120 at the same exposed surface in a similar manner as CD4, albeit it does not require the induction of further conformational changes [14]. The CD4 binding site is highly conserved, but nonetheless not all antibodies targeting the CD4 binding site show broad cross-clade neutralization properties including F105, M12 and M14 for example [15,28–32]. No breakthrough has yet been reported regarding the efficient generation of broadly neutralizing monoclonal antibodies upon immunization of animals with Env antigens [33,34] except for the generation of camelid antibodies. Three heavy chain only camelid specific antibody domains D7, A12 and C8, termed VHH, have been isolated after immunization with gp120. These antibodies compete with CD4 and b12 for gp120 interaction and exert neutralizing activity against primary isolates of subtypes B and C [35].

Here we describe the crystal structure of the camelid VHH D7 and determine the molecular determinants for HIV-1 Env gp120 interaction. Mutagenesis of selected CDR residues abrogate or enhance gp120 interaction in vitro and correlate with the neutralization activity of D7 against the B-clade HIV-1 IIIB thus providing a molecular model for D7-gp120 reactivity.

MATERIALS AND METHODS

D7 purification, crystallization and structure solution.

S. cerevisiae strain VWk18gal1 (CEN-PK102-3A, MATa, leu2-3, ura3, gal1::URA3, MAL-8, MAL3, SUC3) was used for the fermentative production of D7. The VHH D7 gene contains the following amino acid substitutions compared to wild type D7 [35] in framework residues Val5Gln, Ala11Val, Ala61Val, Ala68Asp and Ser79Tyr, which occur naturally in other known VHH structures [36]. The gene was integrated into the S. cerevisiae genome in the rDNA locus [46] and grown as described [47]. VHH D7 was purified from the supernatant using Ni^{2+} affinity chromatography in PBS. A final size exclusion chromatography was performed on a Superdex 200 (GE Healthcare) in a buffer containing 20 mM HEPES pH 7, 0.1 M NaCl. VHH D7 was crystallized using the hanging drop vapor diffusion method at room temperature. Crystals formed by mixing D7 (at 5 mg/ml) with an equal volume of 100 mM sodium cacodylate pH 6, 20 mM magnesium acetate, 1.7 M ammonium sulphate and 19% glycerol. Crystals were flash frozen in liquid nitrogen using 30% glycerol as cryo-protectant. A native dataset was collected to a resolution of 1.5 Å at ESRF beam line (Grenoble, France) BM14. The dataset was processed with MOSFLM [48] and SCALA [49,50]. The crystals belong to space group P212121 with unit cell dimensions of a= 37.37 Å, b= 62.18 Å, c = 62.74 Å containing 1 molecule in the asymmetric unit.

The structure was solved by molecular replacement using the program PHASER [51] and the VHH structure pdb code 1HCV as a search model. The initial model was built using ARPWARP [52] and completed by several cycles of manual rebuilding in COOT [53] and refinement in REFMAC [54] and PHENIX [55] to R_{work}/R_{free} values of 0.1657/0.1935. The final model contains 127 residues, 189 water molecules and 4 sulfates (see table 1). All molecular graphics figures were generated with PYMOL (W Delano; http://www.pymol.org/). Coordinates and structure factors of D7 have been deposited in the Protein Data Bank with accession number 2xa3.

Mutation of VHH D7 and Surface Plasmon Resonance analysis of D7 binding to gp120

Mutations of the VHH D7 sequence [35] were generated by using the quick change mutagenesis kit (Stratagene). The mutations were verified by sequencing. Wild type and mutant VHH D7 proteins were expressed in *E. coli* and purified following the protocol for wild type D7 purification [35]. Gp120 (IIIB) was coupled covalently to channel 2 of a CM5 chip (GE Healthcare) following the manufacturer's instructions. Briefly, the CM5 chip was activated with 50 μL EDC/NHS at a flow rate of 25 μl/min. Adsorption of 100 ml gp120 was carried out in a buffer containing 25 mM sodium acetate pH 5. Deactivation of the surface was achieved with 50 μl diethanolamine. Chanel 1 was treated similar but skipping the step of gp120 adsorption. All binding experiments were performed on a Biacore X (GE Healthcare) in a buffer containing 10 mM HEPES pH 7.5, 100 mM NaCl, 0.05% P20 at a flow rate of 30 μl/min. The association of 90 μl D7 or D7 mutants at concentrations of 25 and 100 nM was recorded for 3 min followed by a dissociation of 15 min. The CM5 chip was regenerated with a 20 ml pulse of 0.1 M glycine pH 2 at a flow rate of 50 μl/min. Data were evaluated with the BiaEvaluation software (GE Healthcare) using simultaneous fit of association and dissociation.

HIV-1 neutralization in TZM-bl cells

HIV-1 neutralization in TZM-bl cells was evaluated using an assay developed previously [56,57,58]. Three-fold serial dilutions of VHH (starting at 20 mg/ml) were prepared in growth medium (DMEM containing 10% FCS) in duplicate wells of opaque 96- well cell culture plates, in a total volume of 50 ml per well. Approximately 200 TCID50 of virus, in 50 ml of growth medium, was added to each well, and the plates were subsequently incubated at 37 °C. After 1 hour of incubation, 10^4 newly trypsinized TZM-bl cells in 100 ml of growth medium containing 30 mg/ml of DEAE-dextran (Sigma-Aldrich) were added to each well. For each plate, six wells containing cells and growth medium only, and six wells containing virus and cells only, were included. The neutralization activity of each VHH was assayed in duplicate. The plates were then incubated at 37 °C for 48 hours and detection of infection of TZM-bl

cells was assayed by measuring luminescence production according to the manufacturer's instructions (Promega). Lysis of cells was allowed to occur for 2 minutes and luminescence (in relative light units; RLU) was then detected using a GloMax 96 Luminometer (Promega). Neutralization was measured as the reduction in RLU in test wells compared to virus control wells after subtraction of background luminescence. The lowest VHH or antibody concentration required to give 50% reduction in RLU (IC50) was determined by fitting the data to a sigmoidal equation using the XLFit 4 software (IDBS).

RESULTS AND DISCUSSION

Structure of VHH D7

The crystal structure of the llama heavy chain antibody fragment VHH D7 was solved by molecular replacement and refined to a resolution of 1.5 Å with an R factor of 16.6% and an R_{free} of 19.4% (table 1). D7 folds into a typical immunoglobulin domain closely resembling known llama VHH structures [36] (Figure 1A). It contains two canonical (CDR1 and CDR2) and a long CDR3 typical for llama VHHs [37] with a non-canonical CDR conformation [38]. CDR3 is composed of 18 residues (Lys^{95} – Tyr^{102}) (Figure 2). The base of CDR3 is well defined and stabilized by multiple main chain and side chain interactions including hydrogen bonds and salt bridges with CDR1 (Ser^{31}-Arg^{97}, Asp^{33}-Lys^{95}, Asp^{33}-Arg^{97} and Asp^{33}-Ser^{100F}) and CDR2

(Ser^{52}-Asp^{100C} and Thr^{56}-Asp^{100C}) (Figure 1B). The extensive inter CDR stabilization suggests a potentially lower flexibility of the CDRs upon binding to gp120. The CD4 binding site antibody b12 employs only one polar (Ser^{30}-Tyr^{53}) and few hydrophobic inter heavy chain CDR contacts [14,39]. However, the tip of the D7 CDR3 (Arg^{100} - Ser^{100B}) is highly mobile evidenced by the lack of continuous main chain density for three residues, including Tyr^{100A} positioned at the apex of CDR3, indicating that their conformational flexibility might be important for gp120 recognition.

Structural comparison of CD4 binding site antibodies

D7 was shown to interfere with CD4 and b12 binding on gp120 and exerts a decent neutralization profile of primary HIV-1 clade B and C isolates [35]. Its neutralization capacity is similar to that of b12, although b12 is more potent and both antibodies neutralize a different spectrum of viruses [35]. MAb b12 employs only the heavy chain to interact with gp120, indicating that a heavy chain only antibody such as the D7 VHH could mimic its interaction with

Table 1. X-ray data collection and refinement statistics

Unit cell dimensions	
a (Å)	37.37
b (Å)	62.18
c (Å)	62.74
Space group	$P2_12_12_1$
Wavelength (Å)	0.974
Resolution (Å)	44.0-1.5
Completeness (%)	94.1 (69.7)
Total reflections	150510
Unique reflections	22695
R_{merge}	0.05 (0.20)
σ	23 (6.5)
Refinement statistics	
Resolution range (Å)	44.2-1.5
Reflections/test set	22363/1135
R-factor	0.1657 (0.1819)
R_{free}	0.1935 (0.2294)
No. of residues	127
No. of water molecules	189
No. of ligand atoms	4 sulfate ions
Average B-factor (Å^2)	13.39
r.m.s.d. from ideal	
Bond lengths (Å)	0.006
Bond angles (deg.)	1.070
Ramachandran statistics	
Most favoured (%)	93.5
Additionally allowed regions (%)	6.5

gp120. All three b12 heavy chain CDRs contact gp120, notably mostly its outer domain [14]. The overlap between the b12 and the CD4 buried surfaces are considerable and both modes of interaction employ aromatic residues to fill a hydrophobic pocket on gp120 (b12 Tyr^{53} of CDR2 and CD4 Phe^{43}) [13,14]. Three other crystal structures of CD4 binding site mAbs are known, namely those of unliganded F105 and m18 Fabs [40,41] and F105 and b13 in complex with gp120 [15]. F105, b13 and m18 interfere with CD4 binding on

gp120, neutralize several HIV-1 strains but exert lower potency in neutralization of primary isolates as compared to b12 [15, 31, 42–44]. The structures of gp120 in complex with F105 and b13 show that it does not suffice to occupy the CD4 binding site in order to exert broad neutralizing activity since the orientation of gp120 recognition is most likely not compatible with binding to the trimeric Env present on virions [15].

CD4 binding site antibodies b12, b13, F105 and m18 display a similar architecture of their CDR3 heavy chains with aromatic residues positioned at the apex of their CDR3 (Figure 3A). This feature led originally to the proposal that this class of antibodies employs aromatic CDR residues to fill the gp120 pocket that is occupied by Phe^{43} in the CD4-bound state [41,45]. Although b12 and b13 point their CDR2 residue Tyr^{53} and Tyr^{52A}, respectively, towards the CD4 binding pocket [14], mAb F105 employs CDR3 Phe^{100A} and Tyr^{100B} within the CD4 binding site [15].

Sequence alignment of D7 with the heavy chain sequences of b12, b13, F105 and m18 reveals aromatic residues within CDR2 and CDR3 (Figure 2). Comparison of the CDR2 loops shows Tyr^{53} at the apex of F105 CDR2, no aromatic residue at the apex of m18 CDR2 and Tyr^{53} and Tyr^{52A} at the apex of b12 and b13 CDR2. The CDR2 of D7 contains Trp^{52A}, which is however not solvent accessible. Instead D7 Trp^{52A} is involved in CDR1 stabilization as observed in another VHH structure [36] (Figure 3B). It is thus unlikely that D7 CDR2 plays a prominent role in gp120 interaction as observed for CDR2 from b12 and b13.

We thus focused our analysis on the CDR3 region as a potential gp120 interaction site. Part of this loop region is highly mobile in the absence of ligand and thus suitable for an induced fit conformation. CDR3 of D7 is tilted 40° towards CDR2 compared to the orientation of the CDR H2/H3 of the other antibodies, whereas the apex of CDR3 is also built by an aromatic residue (Tyr^{100A}) like in b12 (Trp^{100}), F105 (Phe^{100A} and Tyr^{100B}), b13 (Tyr^{100C}) and m18 (Phe^{99}). Notably, b12 employs a number of CDR H3 residues to contact gp120 including Trp^{100} (Figure 3B). However, despite the fact of aromatic residues at the tip of all CDR3 loops, no significant structural

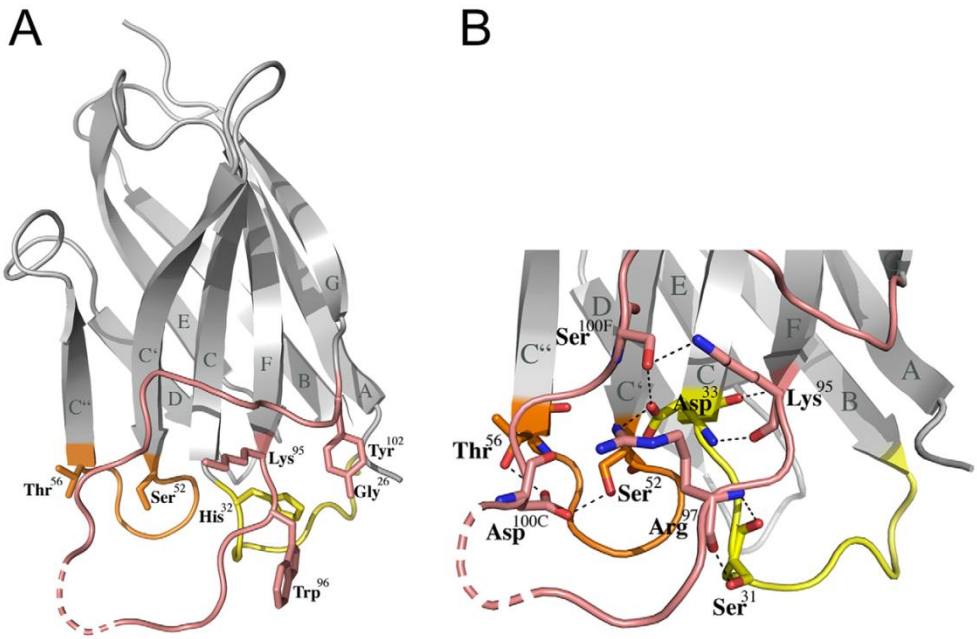

Figure 1. Structure of the llama heavy chain antibody fragment VHH D7. (A) Ribbon representation of D7; the complementarity determining regions (CDR) are highlighted in yellow (CDR1), orange (CDR2) and salmon (CDR3). The first and last residue of each CDR is shown together with the side chain of Trp^{96} critical for gp120 interaction and neutralization. The dotted line indicates CDR3 residues lacking continuous main chain density for residues Arg^{100} to Ser^{100B}. (B) A close-up of the CDR interaction network reveals multiple polar interactions between CDR1 and CDR3 as well as CDR2 and CDR3.

homology between the CDR loops can be observed underlining the differences in mode of gp120 interaction.

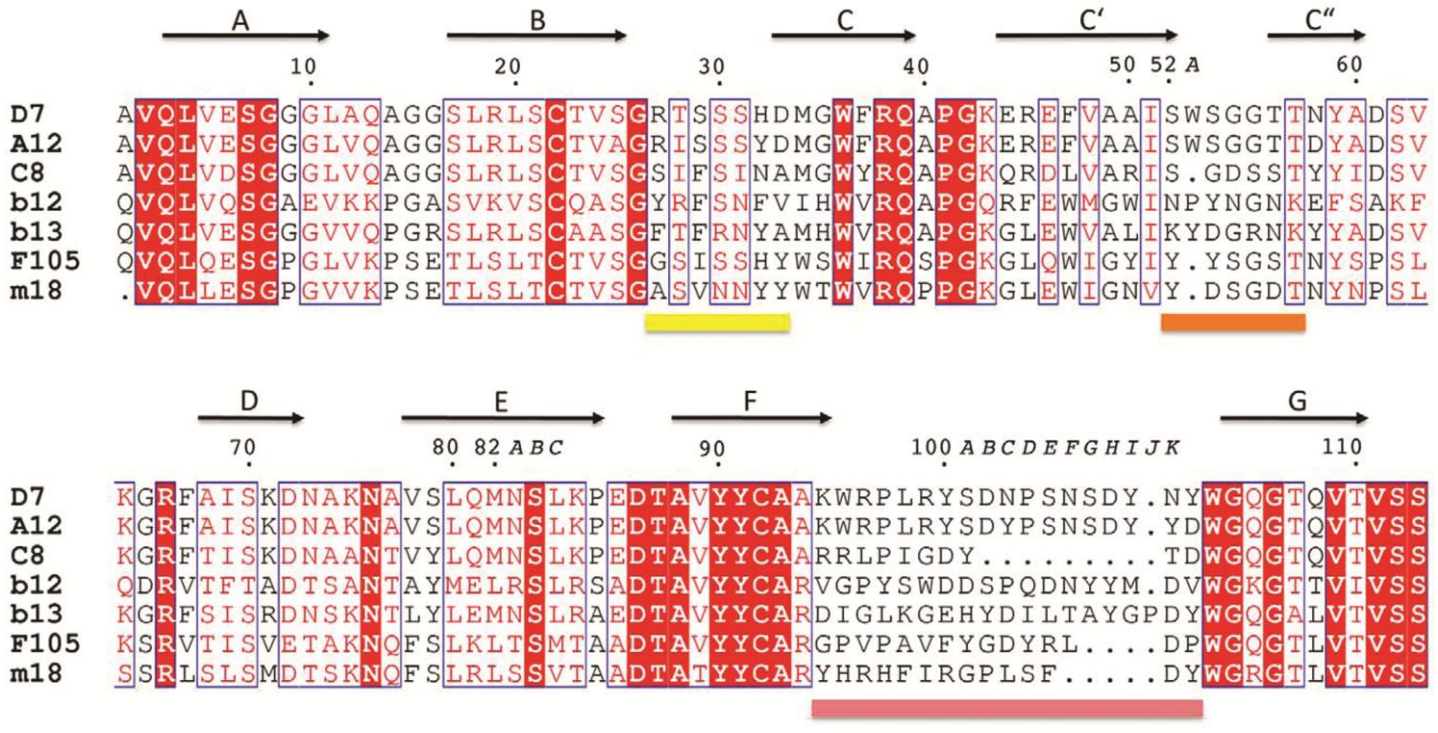

Figure 2. Structure based sequence alignment of D7 with VHH A12 and C8 as well as with the VH domains from the neutralizing antibodies b12, b13, F105 and m18. The residue numbering is according to Chothia and the CDRs are indicated by coloured bars.

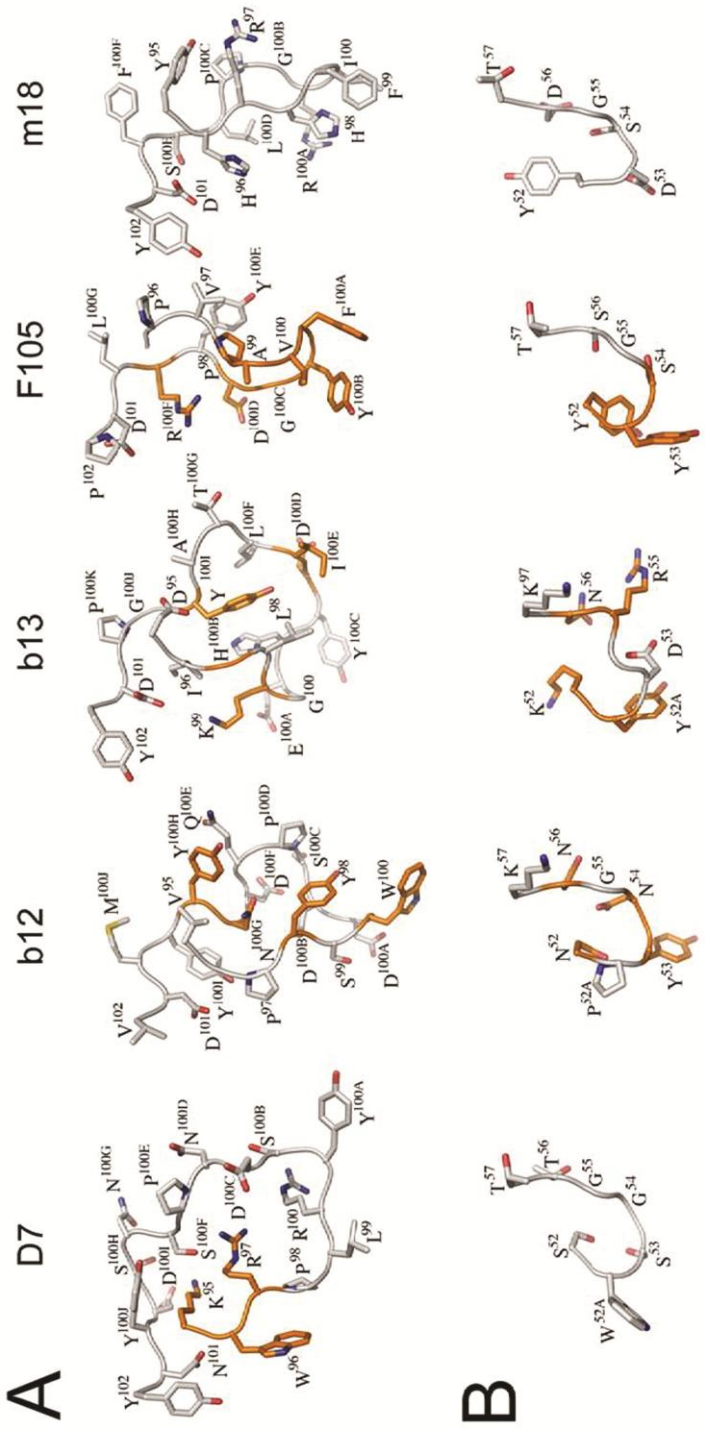

Figure 3. Comparison of CDR2 and CDR3 from D7, b12, b13, F105 and m18 indicates different modes of gp120 interaction. The Ca atoms of the heavy chain variable domains (b12 pdb code 2NY7; F105 pdb code 3HI1; b13 pdb code 3IDX; m18 pdb code 2AJ3) were superimposed and the CDR H2 and H3 are represented in the same orientation. Amino acids are labeled using the one letter code for clarity. (A) All CDR3 loops expose aromatic residues at their apex. (B) The CDR2 of D7 varies from CDR H2 of b12 indicating a different mode of gp120 interaction. Residues implicated in gp120 interaction are highlighted in orange.

Mutational analysis of D7 binding to gp120

We performed mutational analysis within CDR3 to substantiate the role of CDR3 in gp120 interaction by determining the affinity of the D7 mutants in comparison to the wild type using surface plasmon resonance. Mutagenesis of solvent exposed CDR3 residues Lys^{95}Ser, Trp^{96}Ala and Leu^{99}Ala (Figures 2 and 3A; table 2) decreased the affinity of D7 to gp120 (IIIB) significantly to 377, 27 and 69 nM, respectively, compared to 2.9 nM of wild type D7 (table 3). Note that the affinity of D7 to gp120 determined here is ~30 times lower than the previously reported K_D of 0.097 nM [35]. In contrast, mutagenesis of solvent exposed residues Asp^{100C},

Asn^{100D} and the double mutant Asn^{101}Tyr Tyr^{102}Asp (table 2) enhanced the affinity to 0.84, 0.51 and 0.18 nM (table 3). Notably, the double mutation at position 101 and 102 to Tyr and Asp restores the CDR3 sequence of VHH A12 (Figure 2), which has broader neutralization potency than D7 [35]. Mutagenesis of Tyr^{100A} located at the tip of CDR3 (Figure 3A) has no significant effect on binding (table 3). The moderate increase of affinity by mutagenesis of Asp^{100C} and Asn^{100D} might reflect a decrease of the rigidity of the loop since loop stabilizing interactions are affected (table 3 and Figure 1B). This could lead to an improved induced fit upon gp120 binding facilitated by an increased CDR flexibility. The positive effect of the double mutation of

Asn101^{Tyr}102 on interaction together with their close location next to Trp^{96} and Lys^{95} suggest that they affect binding directly. Finally, the strong decrease in affinity upon mutagenesis of residues Lys^{95}, Trp^{96} and Leu^{99} indicate that these residues make crucial contributions to gp120 interaction. The decrease in affinity is mainly due to an increase of the dissociation rate of the D7 mutants (table 3), which suggests a change of interaction between the CDR3 loop and gp120 but no major change of loop conformation. Together, these data implicate that VHH D7 interacts via CDR3 with gp120 and identify Lys^{95}, Trp^{96} and Leu^{99} as key residues for this interaction. Both Lys^{95} and Trp^{96} at the beginning of CDR3 are solvent exposed while Leu^{99} is part of the disordered tip of CDR3, which adds to the conformational freedom of D7 interaction with gp120 (Figure 3A). All three residues are conserved in VHH A12

suggesting that it will utilize its CDR3 to contact gp120 in a similar way (Figure 2). A12 differs only at positions 100D (Tyr instead of Asn) as well as 101 and 102 (Asn, Tyr instead of Tyr, Asp). Further sequence differences that may account for the higher neutralization potency of A12 include CDR1 residues (Thr^{28} is changed to Ile^{28} and His^{32} to Tyr^{32} in A12) and CDR2 residue Asn^{58} (Asp^{58} in A12).

In order to confirm the role of CDR3 and the correlation of gp120 interaction and neutralization, wild type D7 and mutants were tested in a TZM-b1 neutralization assay against HIV-1 IIIB. Comparison of the IC50 values reinforces the importance of CDR3. Mutations that lead to decreased gp120 interaction, namely Ala mutations of Lys^{95} and Trp^{96}, showed a 100-fold increase in IC50, and Leu^{99} revealed a 10-fold increase in IC50 as compared to D7 wild type (table 4). In contrast, mutation of Tyr^{100A} and Asp^{100C} show a modest decrease in the IC50 (table 4) consistent with a slightly increased affinity for gp120 (table 3). The largest positive effect was observed for the double mutant Asn^{101}Tyr^{102}, which shows a ,10-fold increase in affinity (table 3) and a reduction of the IC50 value by a factor of 5 (table 4). This underlines the important role of CDR3 (Figure 4A) for neutralization of HIV-1. Since the CD4 binding site on gp120 is negatively charged [13] CDR3 could provide some complementary basic charge for interaction (Figure 4B). Together these findings indicate that the differences related to CDR3 constitute an important factor accounting for the broader neutralization profile of A12 compared to D7 [35] since both CDR1 and CDR2 are almost identical (Figure 2). It is thus possible that C8 employs different structural principles for gp120 interaction and neutralization [35].

Table 2. Solvent accessible areas of CDR3 D7 residues

Residue	Accessible surface area ($Å^2$)
Lys^{95}	36.56
Trp^{96}	136.34
Leu^{99}	179.13
Tyr^{100A}	220.36
Asp^{100C}	47.52
Asn^{100D}	80.47
Asn^{101}	80.56
Tyr^{102}	52.65

Table 3. Binding affinities of D7 wt and D7 mutants to gp120 IIIB.

Mutation	K_a (10^5 M^{-1} s^{-1})	K_d (10^{-4} s^{-1})	K_D (nM)
Wildtype	1.55± 1.15	5.51± 1.47	2.91± .087
K95S	0.13± 0.10	23.6± 4.90	377± 276
W96A	1.46± 0.46	31.3± 17.1	27.9± 20.5
L99A	0.14± 0.12	50.0± 29.1	69.9± 14.2
Y100aA	2.33± 0.15	3.59± 0.08	1.55± 0.07
D100cA	2.36± 0.13	1.99± 0.38	0.84± 0.12
N100dA	2.52± 0.06	1.28± 0.03	0.51± 0.02
N101Y/Y102D	1.87± 0.08	0.33± 0.27	0.18± 0.15

Table 4. IC50 values of VHH D7 against HIV-1 IIIB in TZM-bl cells.

	IC50 (μg/ml)	IC50 (μg/ml)	IC50 (μg/ml)
mutation	Exp1*	Exp2*	Average
Wildtype	0.066	0.041	0.054
K95S	3.426	2.641	3.0
W96A	2.717	2.428	2.6
L99A	0.817	0.560	0.69
Y100aA	0.028	0.018	0.023
D100cA	0.40	0.014	0.027
N100dA	ND	ND	ND
N101Y/Y102D	0.017	0.005	0.011

* Each experiment was carried out in duplicate wells

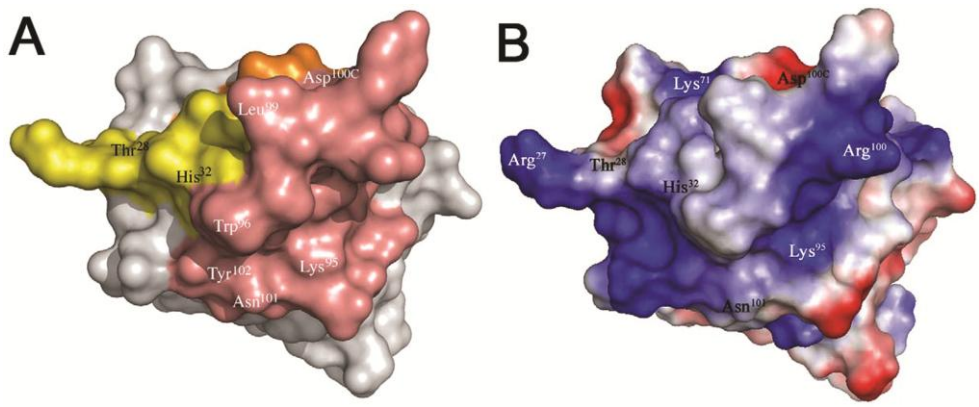

Figure 4. Surface representation of the CDRs. (A) Surface representation of D7 revealing the gp120 docking interface based on gp120 binding and HIV-1 neutralization results. The CDRs are coloured as in figure 1. CDR3 residues affecting gp120 interaction and HIV-1 IIIB neutralization are indicated in white and residue differences between D7 and A12 are labelled in black. (B) electrostatic potential map of the surface generated by the CDRs.

CONCLUSIONS

The conformation of the primary receptor binding site of HIV-1 gp120 reveals a hydrophobic pocket which is the target for Phe^{43} of the natural receptor CD4 [13] and overlaps with the binding sites of neutralizing antibodies b12 [14], b13 and F105 [15]. The structural and mutational data presented here show that D7 does not expose an aromatic residue at its CDR2 and that Tyr^{100A} at the apex of CDR3 does not play a key role in gp120 interaction and HIV-1 IIIB neutralization. Thus D7 might employ different structural principles than b12 to interact with gp120. This is further supported by preliminary results on the D7 interaction with HIV-1 envelope proteins gp140 CN54, gp140 UG37, gp120 IIIB, gp120 YU2 and its modified variant gp120 Ds2, in which an additional S-S (109-428) bridge was introduced, thus closing the cavity below the bridging sheet [14]. VHH D7 binds to gp120 IIIB, gp140 UG37 and gp120 YU2 but is unable to interact with gp120 Ds2 strongly indicating that D7 does not bind to the outer domain, as b12 does (A. Szynol personal communication). This is further supported by antibody recognition of a gp120 escape mutant. Whereas gp120 mutation $Gly^{366}Glu$ results in impaired binding of sCD4 and b12, the binding of D7 was not effected (A. McKnight, personal communication), further corroborating differences in gp120 interaction of b12 and D7. Although we identified Trp^{96} as a key residue for gp120 interaction and neutralization its position and limited extension form the core structure albeit its solvent exposure (table 4) does not conclusively indicate how it could reach into the hydrophobic CD4 binding pocket on gp120. Thus CD4 binding site antibodies might be broadly neutralizing without closely mimicking important molecular details of the CD4-gp120 interaction. Although the structure establishes a firm role for CDR3 and its flexible anchoring in interaction and neutralization activity, structural analysis of gp120 in complex with D7 is required to fully understand the conformational flexibility of both gp120 and D7 in order to exploit this knowledge for a rational vaccine design.

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Chapter 2

Adapted from:

Generation of a Family-specific Phage Library of Llama Single Chain Antibody Fragments That Neutralize HIV-1

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Recently, we described llama antibody fragments (VHH) that can neutralize human immunodeficiency virus, type 1 (HIV-1). These VHH were obtained after selective elution of phages carrying an immune library raised against gp120 of HIV-1 subtype B/C CN54 with soluble CD4. We describe here a new, family specific approach to obtain the largest possible diversity of related VHH that compete with soluble CD4 for binding to the HIV-1 envelope glycoprotein. The creation of this family-specific library of homologous VHH has enabled us to isolate phages carrying similar nucleotide sequences as the parental VHH. These VHH displayed varying binding affinities and neutralization phenotypes to a panel of different strains and subtypes of HIV-1. Sequence analysis of the homologs showed that the C-terminal three amino acids of the CDR3 loop were crucial in determining the specificity of these VHH for different subtype C HIV-1 strains. There was a positive correlation between affinity of VHH binding to gp120 of HIV-1 IIIB and the breadth of neutralization of diverse HIV-1 envelopes. The family-specific approach has therefore allowed us to better understand the interaction of the CD4-binding site antibodies with virus strain specificity and has potential use for the bioengineering of antibodies and HIV-1 vaccine development.

INTRODUCTION

Neutralizing antibodies (NAbs) are an important defense mechanism against virus infections and are the basis for many successful vaccines developed against viruses. In HIV-1-infected patients, NAbs exert a selective effect on virus evolution (1–3). NAbs of HIV-1 target the trimeric envelope (Env) glycoproteins, gp120 and gp41, which mediate binding to the primary receptor, CD4 (4, 5), and co receptor, either CCR5 or CXCR4 (6–11), for virus entry into host cells. However, the elicitation of broad NAbs to HIV-1 *in vivo* is rare due to the enormous global diversity of the HIV-1 Env and due to protection by carbohydrate moieties of neutralization-sensitive epitopes (12, 13). Most neutralizing monoclonal antibodies (mAbs) have been isolated from humans naturally infected with HIV-1 and are usually derived from patients with long term infection (14). The difficulty in eliciting broad NAbs is also demonstrated in immunization programs in humans or animals with HIV-1 Env-based immunogens and represents a major hurdle in the development of an effective humorally based vaccine against HIV-1. Despite these hurdles, passive immunizations with Nabs have been shown to prevent infection and the onset of disease in the macaque model (15–18) and to help in the control of disease progression when introduced post-infection (19). These studies show the importance of further investigation of NAbs for vaccine development and disease control. Identification and characterization of novel broad NAbs may provide additional insights into conserved epitopes that may be targeted for the development of vaccines and entry inhibitors. The engineering of antibodies is therefore an appropriate tool for this purpose.

A small number of broadly neutralizing mAbs have been characterized, which recognize epitopes in the membrane proximal region of gp41 (20–23), the CD4bs (24, 25), the V3 loop of gp120 (25), and a conformational site near the base of the V1/V2 and V3 loops (26). Because the CD4-binding site (CD4bs) must retain some conserved determinants to mediate CD4 binding, NAbs targeting this epitope have the potential to neutralize diverse subtypes of HIV-1. This is confirmed by recent reports that demonstrated the neutralizing activity of broadly neutralizing human sera to be mediated by Abs directed at the CD4bs of gp120 (27–29). We have recently described two llama heavy

chain fragment antibodies (VHH), termed A12 and D7, which compete with sCD4 for Env binding and are able to neutralize different subtypes of HIV-1 (30). To our knowledge, this was the first reported instance where potent cross-subtype neutralizing mAbs against HIV-1 were generated from an immunized animal. Broad NAbs have generally been obtained from natural human HIV-1 infection, after prolonged persistent virus replication and mutation, permitting antibody maturation against multiple conserved epitopes (28). However, the unique properties of llama antibodies allow the isolation of relatively broad Nabs following immunization with Env antigens, giving rise to the A12 and D7 VHH (30) and other VHH from new immunizations still being characterized. The VHH domain consists of the variable region of the heavy chain antibody, which is a unique form of antibodies that members of the Camelidae family produce (31), and they possess all the antigen-binding properties of the complete antibody. Favorable characteristics of VHH that make them suitable for further investigation as mAbs against HIV-1 include their tendency to have affinity and specificity similar to conventional antibodies (32), their longer complementarity-determining regions (CDRs) (33), and their preference for cleft recognition and binding to active sites (34, 35), as well as the potential to format these in multi specific or multivalent constructs (36). Libraries of single chain antibodies are easily produced and have been successfully used for panning against various pathogens (37–39).

Here, we describe a novel method to create a library of related VHH clones that share similar properties to the parental A12/D7 VHH and recognize a similar epitope. The lymphocyte pools from the original immunized llama were mined for VHH variants that were able to recognize the CD4bs of gp120 using molecular techniques, without specific strategies for eluting out CD4bs-specific VHH. A panel of 49 unique VHH was selected from the subfamily library, of which 31 were further characterized.

EXPERIMENTAL PROCEDURES

Recombinant gp120 Antigen Preparation

Recombinant gp120 from HIV-1 IIIB (catalog no. EVA607) was obtained from the Centralized Facility for AIDS Reagents (National Institute for Biological Standards and Control, Potters Bar, UK). A subtype-C recombinant gp120 from HIV-1 92BR025 was expressed in a mammalian cell culture system and purified as described previously (40). Briefly, the envelope gene was amplified by PCR from proviral DNA and cloned into an expression vector with the incorporation of a C-terminal His tag. Recombinant gp120 92BR025 was expressed in 293T cells infected with a T7 RNA polymerase recombinant vaccinia virus (vTF7-3, American Tissue culture Collection number VR-2153). Envelope proteins were harvested 72 h post-transfection and purified using TALON metal affinity resin (Clontech) according to the manufacturer's instructions. Both of these recombinant proteins were then biotinylated using the EZ-Link biotinylation kit (Pierce), according to the manufacturer's instructions. The biotinylated recombinant gp120 was dialyzed with phosphate-buffered saline using Microcon YM-50 centrifugal filter units (Millipore) with a 50-kDa cutoff, and the integrity of the biotinylated roteins was verified in ELISA and Western blots.

A12/D7 Family-specific Phage Library Construction

Total RNA (60 μg) previously isolated from the peripheral blood lymphocyte pools of a llama immunized with recombinant gp120 derived from HIV-1 CN54 was reverse-transcribed into cDNA using random hexamers (SuperScript III, Invitrogen) and cleaned up with QIAquick PCR purification kit (Qiagen). Based on the nucleotide sequences of the A12 and D7 VHH, a unique degenerate reverse primer that extended into the last six codons of the CDR3 loop region was designed to pull out VHH gene fragments with similar sequences through PCR in conjunction with a framework 1-specific primer. The sequences of the A12 and D7 VHH, together with the sequence of the A12/D7 specific primer, are shown in Figure 1. The A12/D7 CDR3 sequences do not share any homology with other known CD4bs-specific antibodies using a BLAST search. The PCR amplification was carried out with

Expand High Fidelity (Roche Applied Science) using the following program: 94 °C for 2 min, followed by 28 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 7 min. The PCR was limited to 28 cycles to prevent oversaturation of the amplified products, and a 350-bp band was excised after separation on an agarose gel. Following restriction enzyme digestion with BstEII and SfiI and gel purification, the digested DNA fragments were ligated into a phagemid vector (pAX50) for display on filamentous bacteriophage and electrotransformed into *E. coli* TG1 competent cells as described previously (41). The transformed cells were titrated on agar plates to determine the library size, and a colony PCR was performed on a selection of colonies to determine the presence of DNA inserts in the vector.

Bio-panning and Retrieval of Binders

The family-specific phage library displaying the cloned VHH repertoire was preincubated with four dilutions (0.01–10 nM) of biotinylated recombinant gp120 antigens in 0.2% casein on a reblocked microtiter plate for 2 h. The binding of phages to antigens in solution reduced the avidity effects of the binding. Phages bound to the biotinylated antigens were subsequently captured by incubating the mixture for 30 min on immobilized neutravidin (Sigma) or on D7324 (Aalto Bio Reagents, Dublin, Ireland) that was precoated overnight in 96-well Maxisorp plates (Nalgene,Hereford, UK). D7324 is a sheep polyclonal antibody raised against a conserved motif in the C terminus of gp120. Intensive washing with 0.05% Tween in phosphate-buffered saline (PBS-T) was carried out to remove any unbound phage. As the family-specific phage library was assumed to contain only phages that recognize the CD4bs, a general trypsin (1 mg/ml) elution was used to harvest the bound phages instead of a competitive sCD4 elution originally used to isolate the parental A12/D7 VHH. The trypsin was then neutralized with 210 μM trypsin blocker acetylbenzenesulfonyl fluoride (Sigma). Selections where a larger number of clones were eluted than blank controls, while keeping background to the minimum, were taken forward to a second round of panning. Selections were carried out for two rounds. Genes from selected VHH were recloned in an expression vector (pAX51), which lacks the phage-derived gene 3. The VHH is produced with a C-terminal hexahistidine tail under the control of the *lac*

promoter (35). Following transformation into *E. coli* TG1 cells, individual colonies were picked and cultivated in 2X TY medium containing 100 μg/ml ampicillin and 0.1% glucose. Expression was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h as soluble periplasmic proteins. VHH chosen for further characterization were then purified by immobilized metal affinity chromatography using TALON metal affinity resin (Clontech) and then dialyzed against phosphate buffered saline.

Competition ELISA

An ELISA was carried out to determine whether the selected VHH were able to compete with sCD4 for binding to gp120, and performed as described previously (30). Briefly, 10 μg/ml recombinant sCD4 containing the D1–D3 regions of CD4, obtained from the Centralized Facility for AIDS Reagents (National Institute for Biological Standards and Control, Potters Bar, UK), was coated overnight on 96-well Maxisorp plates. After blocking with 4% skim milk, 1 μg/ml gp120 IIIB was preincubated for 1 h with serial dilutions of VHH and negative control VHH and then subsequently added onto the plates for 1 h. The mAb b12, which targets the CD4bs of gp120, was included for comparison. Bound gp120 was detected with 10 μg/ml of the sheep polyclonal antibody D7324 (Aalto BioReagents) and then with 0.5 μg/ml horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody (Dako, Denmark). SureBlue TMB microwell substrate (Kirkegaard & Perry Laboratories) was added and left to develop for 30 min, after which the reaction was stopped with 1 M HCl. The absorbance was measured at 450 nm.

Surface Plasmon Resonance

The kinetic binding parameters were determined with Biacore®. gp120 IIIB was diluted in 10 mM sodium acetate, pH 4.0, to a concentration of 15 μg/ml and injected over the surface at a flow rate of 5 μl/min for coupling via free amines to the CM5 sensor chip. Excess activated groups were blocked using a 7-min injection of 1 M ethanolamine, pH 8.5, at a flow rate of 5 μl/min. Purified VHH was diluted in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% P20 surfactant) to 75, 50, 40, 30, and 20 nM, and the samples were injected for 2 min. A reference surface where no

compound was immobilized was also included. Association was measured for 3 min and dissociation for 15 min. Regeneration was achieved by washing with 10 mM HCl for 3 min. The kinetic constants (*i.e.* the second-order rate constant for the association, k_a, and the first-order rate constant for the dissociation, k_d) were computed from the sensograms using the BIAEvaluation software (1:1 interaction), and the equilibrium dissociation constant (K_D) was calculated from k_d/k_a.

Mutation of VHH

Site-directed mutagenesis of the YYD[102] motif at the C terminus of the CDR3 was performed by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) in conjunction with the appropriate primers according to the manufacturer's instructions. The mutations were verified by sequencing, followed by the expression and purification of VHH as described above, and are listed in Table 4. These were tested for binding in ELISA and for its neutralization potency in the TZM-bl assay.

Viruses

HIV-1 IIIB was obtained from the Centralized Facility for AIDS reagents (National Institute for Biological Standards and Control, Potters Bar, UK) and propagated in H9 cells. The subtype C Env gp120 clones, C222 and 92BR025 (42), were introduced into the pHXB2Δenv vector (43) to produce replication-competent chimeric viruses after transfection in 293T cells. The QH0692.42, PVO.4, and ZM214M clones are part of the subtype B and C HIV-1 Reference Panels of Env Clones (44, 45) and were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. These HIV-1 envelope pseudotyped viruses were produced in 293T cells by cotransfection with the pSG3Δenv plasmid.

HIV-1 Neutralization Assays

The neutralizing ability of the VHH was tested against a panel of HIV-1 viruses using a luciferase- based assay in TZM-bl cells (46–48), which was obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health, from J. C. Kappes, X. Wu, and

Tranzyme, Inc. This assay measures the reduction in levels of Tat-driven luciferase reporter gene expression following a single round of virus infection in TZM-bl cells. Briefly, 3-fold serial dilutions of purified VHH starting from 25 μg/ml were performed in duplicate in 10% (v/v) fetal calf serum-supplemented Dulbecco's modified Eagle's growth medium (Invitrogen) using 96-well flat-bottom culture plates (Nuclon, Nunc). 200 $TCID_{50}$ titers of virus were then added to each well, and the plates were incubated for 1 h at 37 °C. TZM-bl cells were subsequently added (1x 10^4 cells/well) in growth medium supplemented with DEAE-dextran (Sigma) at a final concentration of 11 μg/ml. Assay controls included replicate wells of TZM-bl cells alone (background control) and TZM-bl cells with virus assayed (virus control). No virus inactivation was observed with an irrelevant negative control VHH. Following 48 h incubation at 37 °C, all but 100 μl of the assay medium was removed, and 100μl of Bright-Glo luciferase reagent (Promega, Madison, WI) was added to each well. The cells were allowed to lyse for 2 min, and the luminescence was measured using a luminometer. The 50% inhibitory concentration (IC50) titers were calculated as the VHH concentration that achieved a 50% reduction in relative luminescence units compared with the virus control relative luminescence units, after subtraction of the background control relative luminescence units from both values. The calculations were performed using the XLFit4 software (ID Business Solutions, Guildford, UK), and the assay was performed on at least two separate occasions.

RESULTS

Construction of the A12/D7 Family-specific Phage Display Library

Both the A12 and D7 VHH were isolated from a llama that was immunized with recombinant gp120 derived from HIV-1 CN54 (subtype B'/C, CRF07_BC) and were able to cross-neutralize various subtypes and strains of HIV-1 (30). Because the CDR3 possesses the greatest flexibility and conformational variability of the CDRs, it could have the greatest influence on antigen binding (49). As A12 and D7 share similar sequences, we designed an A12/D7-specific degenerate primer that recognized the C-terminal stretch of nucleotides in the CDR3 loop and the first four (conserved) amino acids of framework 4 of A12/D7 to amplify all the affinity- saturated antibodies that share sequences homologous to the A12 and D7 VHH. RNA extracted from the peripheral blood lymphocyte pool of the original immunized llama was amplified by PCR with the A12/D7-specific primer in conjunction with a primer to a highly conserved framework 1 region, and an A12/D7 family-specific phage display library was generated (Figure 1). The size of this library was estimated to be 2.0 x 10^6 clones, and the proportion of clones containing inserts was determined to be 91% (data not shown). To our knowledge, this is the first published instance to describe the creation of a family-specific library containing homologous single chain antibodies recognizing a specific epitope.

Selection and Screening of VHH

Biopanning was carried out on two different antigens, gp120 IIIB (subtype B) and gp120 92BR025 (subtype C), in parallel. As the A12/D7 VHH was originally panned on gp120 IIIB, adding a subtype C Env may promote the selection of VHH with cross-subtype recognition properties. The recombinant gp120 92BR025 was cloned directly from a primary isolate and expressed in a mammalian expression system. To remove avidity effects, the phages were allowed to bind to biotinylated gp120 in solution, before capturing the bound phages on either neutravidin or the anti gp120 Ab D7324-coated plates. Although the original A12 and D7 VHH were competitively eluted with

(A)

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 FR1 primer →
A12          GCGGTGCAGCTGGTGGAGTCTGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAGACTC 60
D7           GCGGTGCAGCTGGTGGAGTCTGGGGGAGGATTGGCGCAGGCTGGGGGCTCTCTGAGACTC 60

A12          TCCTGTACAGCCTCTGGACGCATCAGCAGTAGCTATGATATGGGCTGGTTCCGCCAGGCT 120
D7           TCCTGTACAGTTTCTGGACGCACCAGTAGTAGCCATGATATGGGCTGGTTCCGCCAGGCT 120

A12          CCAGGGAAGGAGCGTGAGTTTGTAGCGGCTATTAGTTGGAGTGGTGGTACCACAGACTAT 180
D7           CCAGGAAAGGAGCGTGAGTTTGTAGCGGCTATTAGCTGGAGTGGTGGTACCACAAACTAT 180

A12          GCAGACTCCGTGAAGGGGCGATTCGCCATCTCCAAAGACAACGCCAAGAACGCAGTGTCC 240
D7           GCAGACTCCGTGAAGGGGCGATTCGCCATCTCCAAAGACAACGCCAAGAACGCAGTCTCC 240

A12          CTGCAAATGAACAGCCTGAAACCCGAGGACACGGCCGTTTATTACTGTGCAGCTAAGTGG 300
D7           CTGCAAATGAACAGCCTGAAACCTGAGGACACGGCCGTTTATTACTGTGCAGCTAAGTGG 300

A12          CGACCGCTACGTTATAGTGACTACCCTTCGAATTCGGATTACTACGACTGGGGCCAGGGG 360
D7           CGACCGCTACGTTATAGTGATAACCCTTCGAATTCAGATTATAACTACTGGGGCCAGGGG 360
                                        ← A12/D7 primer

A12          ACCCAGGTCACCGTCTCCTCA 381
D7           ACCCAGGTCACCGTCTCCTCA 381
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(B)

A12/D7-specific primer:

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5'- TGAGGAGACGGTGACCTGGGTCCCCTGGCCCCAGTMGTWRTAATCYGAATTCG -3'
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Figure 1. Nucleotide sequences of A12 and D7 (A) and the A12/D7-specific primer (B) are shown with the CDR3loop-encoding region shaded in gray. A unique degenerate reverse primer was designed (B) to extend into the CDR3 region and is complementary to the underlined sequence in A.

soluble CD4 to obtain antibodies that can inhibit CD4 binding to gp120, a general elution method using trypsin was used here to elute all bound phages, as all the VHH in the family-specific library were assumed to be related to A12/D7 and therefore specific for the CD4bs on gp120. Two rounds of selections were carried out on both antigens in parallel, and the outputs that gave the best enrichment of binders from the optimum concentration of antigen were brought forward to be subcloned into an expression vector providing a C-terminal His_6 tag for purification. Ninety four clones were picked from each output, a summary of which is shown in Table 1. A restriction analysis of the selected clones showed low variations in diversity, which was expected as they were all part of the same family (data not shown).

The clones were further characterized by sequencing. Out of the total 376 clones picked, there were 49 unique amino acid sequences. Although two

Table 1. Summary of outputs from selections

Outputs	1st round	2nd round
2B10	0.1 nM gp120 IIIB	1 pM gp120 IIIB
2B12	0.01 nM gp120 IIIB	1 pM gp120 IIIB
2C7	10 nM gp120 92BR025	10 pM 92BR025
2C9	1 nM gp120 92BR025	10 pM 92BR025

A summary of the selection conditions used in the biopanning process is shown. Two outputs of 94 clones each were collected, derived from selections with different antigen concentrations, giving a total of 376 clones.

different antigens were used in the biopanning, identical clones were found in both selection strategies. All newly isolated VHH showed high sequence homology to the parental A12/D7 VHH, but variations in the sequences were observed in the framework regions, as well as the complementary-determining regions of the VHH.

Selected VHH Inhibit Binding of sCD4 to gp120

As the parental A12 and D7 VHH competed with sCD4 for binding to gp120 in both the ELISA and the surface plasmon resonance assays (30), a competition ELISA was carried out to determine whether the newly selected cousins from the family-specific VHH isolation recognized a similar epitope on gp120. A fixed amount of gp120 IIIB was first allowed to bind to titrated amounts of VHH. The ability of the bound VHH to inhibit the interaction of gp120 with CD4 coated onto the solid phase was then measured. Fifteen different VHH from the different outputs representing a range of different affinities were tested in this assay, together with an irrelevant VHH as negative control. The parental D7 VHH and a CD4bs human mAb, b12 (50), were also included in this assay. From Figure 2, all the members of the family- specific VHH, together with the D7 VHH and mAb b12, are shown to compete with sCD4 for binding onto gp120 in a dose dependent manner. In contrast, the irrelevant negative control VHH had no effect on sCD4 binding. Hence, although a specific elution strategy was not used in the biopanning, all of the selected VHH were found to recognize gp120 in a similar fashion to A12/D7. The creation of the A12/D7 family-specific VHH was therefore successful.

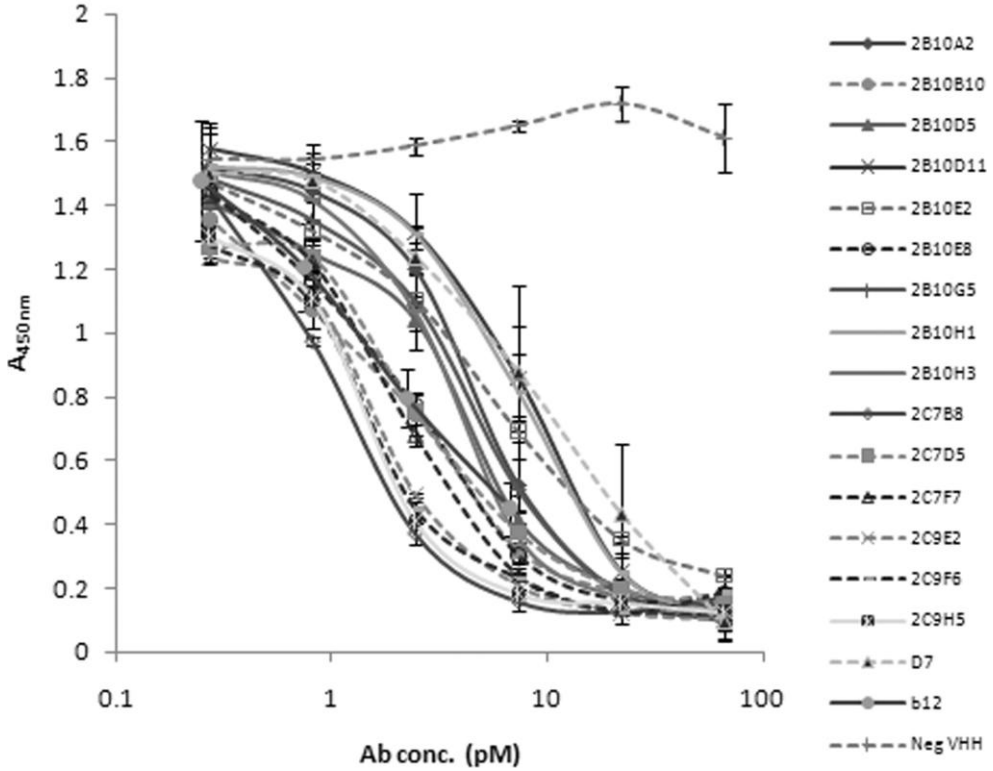

Figure 2. VHH inhibition of sCD4 binding to gp120. A selection of 15 VHH from the A12/D7 family-specific library was tested for their ability to inhibit sCD4 binding to gp120, together with the parental D7 VHH and the mAb b12. All of them were found to inhibit sCD4 binding in a dose-dependent manner. An irrelevant VHH was used as a negative control, and this did not affect sCD4 binding.

Variations in Neutralization Potencies

Thirty one different VHH from the family-specific library were brought forward for evaluation in HIV-1 neutralization assays. The VHH were selected based on differences in their amino acid sequences, especially in the CDR3 loop regions, and they included the 15 VHH tested for CD4 inhibition (see above). The parental A12 and D7 VHH had been tested in the TZM-bl assay against a broad panel of T-cell line adapted viruses, cloned Env pseudotyped viruses, as well as cloned Env replication-competent chimeric viruses, and A12 was also shown to have neutralizing activity in a peripheral blood mononuclear cell assay (30). To study the 31 VHH in detail, the number of viruses tested

was narrowed down to a smaller panel of six viruses, which consisted of three subtype B viruses (IIIB, QHO692.42, and PVO.4) and three subtype C viruses (C222, 92BR025.C1, and ZM214M.PL15). Neutralization was assayed using the TZM-bl cell line. TZM-bl cells contain a Tat-dependent luciferase reporter system, which is only induced after HIV-1 infection. Neutralization by VHH was measured as the reduction of relative light units emitted compared with virus without VHH. TZM-bl cells express CD4 and the coreceptors, CXCR4 and CCR5, and thus they are sensitive to infection by all HIV-1 strains. The VHH concentration required to achieve 50% reduction of infectivity was determined, and the results are shown in Table 2. The parental A12 and D7 were included for comparison.

All VHH tested displayed similar neutralizing activity against the three subtype B viruses assayed; they were highly effective against IIIB (IC50 < 1 μg/ml), moderately potent against QHO692.42 (10 < IC50 < 25 μg/ml), and ineffective against PVO.4 (IC50 > 25 μg/ml). However, a different pattern emerged when the VHH were tested on the subtype C viruses. None of the VHH were able to neutralize ZM214M.PL15, but they showed marked differences in their ability to neutralize C222 and 92BR025.C1. Based on their IC50 values for C222 and 92BR025.C1, the VHH can be grouped into three categories named Broad, Intermediate, and Narrow. VHH in the Broad category were able to neutralize both C222 and 92BR025.C1 to high potency (IC50 < 1 and < 5 μg/ml, respectively), whereas VHH in the Intermediate category were able to neutralize C222 (0.5 < IC50 < 5.0 μg/ml) but not 92BR025.C1 (IC50 > 25 μg/ml).

Finally, VHH in the Narrow category were unable to neutralize both C222 and 92BR025.C1 (IC50 > 25 μg/ml). Within the Broad category, the ranges of IC50 values for C222 and 92BR025.C1 were ~ 10- and 2-fold, respectively. The parental A12 falls into the Broad category, whereas D7 falls into the Narrow category.

Table 2. VHH IC50 (µg/ml) titers against HIV-1.

	VHH	VHH Neutralization shown as IC_{50} (µg/ml)					
		Subtype B			Subtype C		
		IIIB	QH0692.42	PVO.4	C222	92BR025.C1	ZM214M.PL15
Broad	2B10A2	0.07	19.73	>25	0.13	2.01	>25
	2B10C2	0.16	18.66	>25	0.12	1.57	>25
	2B10D5	0.57	19.63	>25	0.96	1.95	>25
	2B10D7	0.12	21.63	>25	0.20	1.42	>25
	2B10D11	0.18	15.48	>25	0.36	4.47	>25
	2B10G5	0.40	16.10	>25	0.04	0.28	>25
	2B10H1	0.29	17.21	>25	0.08	0.74	>25
	2B10H3	0.29	19.14	>25	0.06	0.39	>25
	2C7B8	0.15	10.78	>25	0.27	0.78	>25
	2C7D2	0.17	20.71	>25	0.28	0.60	>25
	2C7E3	0.07	12.16	>25	0.13	0.48	>25
	2C7G11	0.16	16.08	>25	0.24	1.76	>25
	2C9A8	0.30	nd	>25	0.41	2.40	>25
	2C9B11	0.24	nd	>25	0.30	3.41	>25
	2C9C5	0.12	nd	>25	0.16	1.23	>25
	2C9D9	0.37	nd	>25	0.61	3.64	>25
	2C9E4	0.35	nd	>25	0.50	1.76	>25
	2C9E7	0.30	20.87	>25	0.34	0.90	>25
	2C9F1	0.13	19.31	>25	0.48	0.95	>25
	2C9F10	0.13	18.70	>25	0.16	0.61	>25
	2C9H5	0.17	17.28	>25	0.19	0.73	>25
Inter-mediate	2C7F7	0.34	17.31	>25	4.53	>25	>25
	2C7H5	0.12	16.50	>25	10.20	>25	>25
	2C9E2	0.20	19.39	>25	4.08	>25	>25
Narrow	2B10B10	0.54	19.40	>25	>25	>25	>25
	2B10E2	0.46	15.90	>25	>25	>25	>25
	2B10E8	0.49	18.54	>25	>25	>25	>25
	2C7D1	0.24	16.29	>25	>25	>25	>25
	2C7D5	0.13	20.04	>25	>25	>25	>25
	2C7E11	0.21	17.27	>25	>25	>25	>25
	2C9F6	0.18	14.54	>25	>25	>25	>25
	A12	0.09	13.00	>25	0.07	0.23	>25
	D7	0.30	17.00	>25	>25	>25	>25

VHH from the family-specific library, together with the parental A12 and D7, were tested for their potency in neutralization against the indicated viruses as described in the text. The VHH were categorized into Broad, Intermediate, or Narrow, according to their potency against C222 and 92BR025.C1. To aid comprehension, the titers have been shaded, with darker colors indicating more potent neutralization. nd indicates not determined.

Sequence Analysis of the CDRs

All 31 VHH from the family-specific library possess unique sequences with variations seen within the framework regions as well as in the CDRs. To understand the molecular basis of the variation in neutralizing activity, we studied the amino acid sequences of the CDRs as they are crucial for antigen binding and specificity (51). The amino acid positions of the CDRs are defined using Chothia numbering as follows: 26–32 for CDR1, 52–56 for CDR2, and 95–102 for CDR3 (52) and are grouped according to their three neutralizing categories (Table 3). Certain residues within the CDRs were found to show mutations in high frequencies. These are residues 28, 31, and 32 within CDR1, residue 56 in CDR2, and residues 100D, 101, and 102 in CDR3. These "hot spot" residues, except for 101 and 102 in CDR3, are also found in VHH with Broad neutralizing activity and are therefore unlikely to compromise the neutralization ability of the VHH on the viruses tested, but might affect the range of IC50 values observed for C222 and 92BR025.C1 within the Broad category. When analyzing residues 101 and 102, a distinct YYD^{102} motif at the C-terminal end of the CDR3 loop is conserved within the Broad neutralizer category but absent from the Narrow neutralizer category. Mutation of this YYD^{102} motif to YYY, YNY, or YND seemed to hamper neutralizing potency against the subtype C viruses C222 and 92BR025.C1. For instance, five of the eight Narrow neutralizers (2B10E8, 2B10E2, 2C9F6, 2B10B10, and 2C7D1) and two of the three Intermediate neutralizers (2C7H5 and 2C9E2) have CDR sequences that are identical to those found in one or more Broad neutralizers, except for residues 101 (Tyr to Asn substitution) and/or 102 (Asp to Tyr substitution). Paired groups marked with an asterisk in Table 3 have an Y101N substitution, whereas VHH marked with a double-plus sign have Y101N and D102Y mutations, and those marked with a plus sign have Y101N and/or D102Y mutations.

To confirm that the YYD^{102} motif is crucial for the Broad neutralizing ability of the VHH, we extended the sequence analysis to include framework sequences and searched for VHH cousins with identical sequences but varying in the YYD^{102} motif. In Figure 3*A*, the sequences of 2C7D2 (a Broadly neutralizing VHH) and 2C9E2 (an Intermediate neutralizing VHH) are

identical except for a single point mutation (D102Y) to produce a **YYY**[102] motif. Likewise, in Figure 3*B*, a double mutation from YYD[102] to **YNY**[102] is the only difference between the two VHH 2B10G5 and 2C7D1, which display a Broad and Narrow phenotype, respectively. VHH with a single Y101N mutation to produce the **YND**[102] motif can display either an Intermediate or Narrow phenotype, as demonstrated by 2C7H5 and 2C7E11 in Figure 3*C*. These two VHH contain a few other mutations in their sequences, but because those residues are also found in other VHH within the Broad category, they are unlikely to have an impact on neutralizing activity. The YYY[102], YNY[102], and YND[102] motifs are therefore associated with a less broad neutralizing potential, and many more VHH variants within the family-specific library exist to support this conclusion. Interestingly, one variant VHH 2C7F7 possessed the YYD[102] motif but fell under the Intermediate category. This VHH (Figure 3*D*) contains three unique mutations (H32F, M34L, and A40G) that are not found in any other VHH within the family and involve changes in the hydrophobicity of the residues. For example, a unique phenylalanine residue that is non-polar and very hydrophobic had replaced the polar histidine or tyrosine residues that are usually found in position 32 within CDR1. Together, these cumulative mutations may have an effect on antigen binding and thus compromise the potential for the Broad phenotype conferred by the YYD102 motif.

Mutations in the YYD102 Motif

To confirm the significance of the YYD[102] motif at the end of the CDR3 and its associated effect on the recognition and neutralization of HIV-1, eight different mutations were generated in the YYD[102] and YNY[102] motif of the parental A12 and D7, respectively. The residues in positions 101 and 102 were mutated to determine the contribution of each residue, and a list of the mutants is shown in Table 4. Mutations in either or both these residues did not abrogate binding to gp120 IIIB in ELISA, and all mutants were still able to neutralize HIV-1 IIIB to high potency (IC50 < 1 μg/ml). Mutants 1–3 are point mutations of A12 where Tyr[101] was changed into Ala[101], Asn[101], and Gln[101], respectively. When compared with A12, mutants 1 and 2 lost the ability to neutralize both C222 and 92BR025.C1 with just a single amino acid change.

Table 3. Sequence comparison of the CDRs.

VHH	CDR1	CDR2	CDR3
Consensus	*26 32* . . GRISSSH	*52ABC3456* . SWSGGTTD	*95 100ABCDEFGHIJ 102* . . . KWRPLRYSDNPSNSD**YYD**
Broad neutralizers			
2B10D5*	--T----	--------	----------
2B10C2*	--T----	--------	----------
2C9A8	--T----	--------	-------N----------
2C9B11	--T----	--------	-------N----------
2B10A2	--T----	-------N	----------
2B10D11	-------	--------	----------
2C7E3	-------	-------N	---------S----------
2C9C5	-------	-------N	---------S----------
2C9F10	-------	-------N	---------S----------
2C7D2†	-------	--------	---------D----------
2B10G5†	-------	--------	---------D----------
2C9E7†	-------	--------	---------D----------
2B10H3	-------	----A---	---------D----------
2C7B8	------Y	-----A--	---------Y----------
2B10D7‡	------Y	--------	---------Y----------
2B10H1‡	------Y	--------	---------Y----------
2C9F1	------Y	------K-	---------Y----------
2C9H5	------Y	---S----	---------Y----------
2C7G11	------Y	--------	---------S----------
2C9D9	-----MY	-------A	---------D----------
2C9E4	-----MY	-------A	---------D----------
A12	------Y	-------	---------Y----------
Intermediate neutralizers			
2C7H5†	-------	--------	---------D---------**-N-**
2C9E2†	-------	--------	---------D---------**--Y**
2C7F7	--T---F	--------	---------D----------
Narrow neutralizers			
2B10E8*	--T----	--------	----------**-N-**
2C7E11	--V----	--------	--------E---------**-N-**
2B10E2‡	------Y	--------	---------Y---------**-NY**
2C9F6	------Y	--------	---------Y---------**--Y**
2B10B10‡	------Y	--------	---------Y---------**-NY**
2C7D1	-------	--------	---------D---------**-NY**
2C7D5	--L--MY	-----S-T	----------**-N-**
D7	--T----	-------N	----------**-NY**

The VHH are grouped into the three different neutralization categories, and the amino acid sequences of their CDRs are aligned and compared. The consensus sequence with Chothia numbering to denote the CDR position is shown in the top row. Residues that are identical to the consensus sequence are shown in dashes. Variants of the YYD102 motif at the end of the CDR3 are shown in boldface type, and the YYD102 motif is underlined. The symbols *, †, and ‡ are used to denote groups of VHH with similar CDR sequences but with variations only in the YYD102 motif. (* Y101N substitution; ‡ Y101N and D102Y mutations; † Y101N and/or D102Y mutations.)

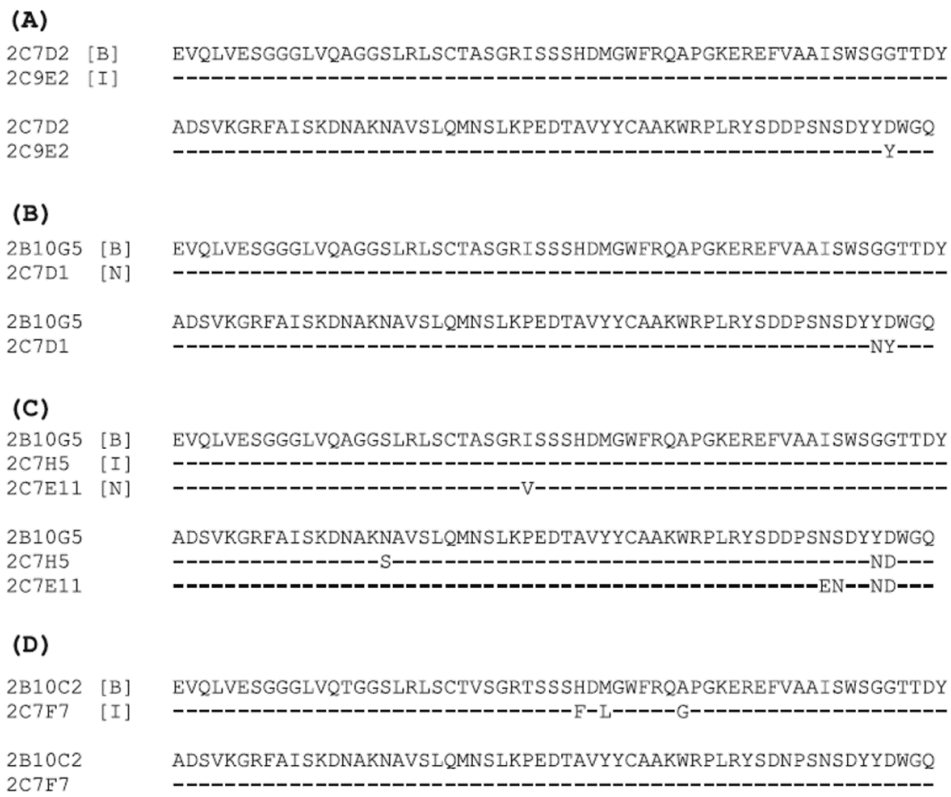

Figure 3. Sequence comparison of VHH pairs. Alignments of paired amino acid sequences of closely related VHH that display different neutralization phenotypes were compared. Identical residues are denoted in dashes, and the neutralization category of each VHH is denoted in square brackets (B, Broad; I, Intermediate; N, Narrow). A, YYD to YYY mutation resulted in a change from Broad to Intermediate category. B, double mutation from YYD to YNY resulted in a change from Broad to Narrow category. C, switch from YYD to YND can result in a change from Broad to Intermediate and Narrow category. Mutations in the other residues are unlikely to significantly influence the neutralization phenotype as these amino acid substitutions are present in other VHH in the Broad category. D, despite having a YYD motif in 2C7F7, a unique three-point mutation resulted in a change from Broad to Intermediate category. The H32F mutation is within the CDR1.

Mutant 3 lost the ability to neutralize 92BR025.C1 and a 200-fold drop in potency against C222. Mutants 4 and 5 are point mutants of A12 where Asp102 was changed into Ala102 and Glu102, respectively. Mutant 5 retained

Table 4. Characterization of A12/D7 mutants.

Mutant No.	Parent	Motif 100J-102	ELISA	Neutralization IC50 (μg/ml)			Category
				IIIB	C222	92BR025	
A12	-	YYD	Yes	0.01	0.03	0.25	Broad
1	A12	YAD	Yes	0.2	>25	>25	Narrow
2	A12	YND	Yes	0.12	>25	>25	Narrow
3	A12	YQD	Yes	0.08	6.63	>25	Intermediate
4	A12	YYA	Yes	0.04	0.97	>25	Intermediate
5	A12	YYE	Yes	0.02	0.09	0.71	Broad
6	A12	YNY (D7-like)	Yes	0.88	>25	>25	Narrow
7	D7	YND	Yes	0.11	>25	>25	Narrow
8	D7	YYD (A12-like)	Yes	0.04	0.05	1.27	Broad
D7	-	YNY	Yes	0.07	>25	>25	Narrow

Mutations in residues 101 and 102 were carried out on the parental A12 and D7 VHH. All the mutants were able to bind to IIIB gp120 in ELISA and neutralized HIV-1 IIIB with high potency. Mutants 1 and 2 lost the ability to neutralize both C222 and 92BR025 with just a point mutation change in residue 101. Mutant 5 retained the ability to neutralize both C222 and 92BR025 despite a D102E mutation. Mutants 6 and 8 are double back mutations where the YYD102 and YNY102 motifs were inter-switched, which resulted in a corresponding change in the neutralization phenotype. Residues 101 and 102 at the end of the CDR3 loop are therefore important for the subtype C Env specificity tested here.

the ability to neutralize both viruses, and mutant 4 lost the ability to neutralize 92BR025.C1 only. Therefore, a single point mutation in either residue 101 or 102 was sufficient to cause a phenotype change. It would also appear that residue 101 is more important in discriminating between the two viruses than residue 102. Mutants 6 and 8 contain two double back mutations where the YYD^{102} and YNY^{102} motifs of both A12 and D7 were inter-switched. These resulted in a corresponding neutralization phenotype change from Broad to Narrow and vice versa, thus confirming that the final two residues of the CDR3 loop are important in the neutralization of the subtype C viruses tested here.

Table 5. Binding affinities of VHH.

VHH	K_a	K_d	K_D	Motif	Neutralization category
	10^5 M^{-1} s^{-1}	10^{-4} s^{-1}	nM		
A12	2.73	0.298	0.10	YYD	Broad
2B10H1	1.48	0.252	0.17	YYD	Broad
2B10H3	1.64	0.312	0.19	YYD	Broad
2B10H5	1.47	0.309	0.21	YYD	Broad
2B10D11	1.12	0.247	0.22	YYD	Broad
2B10C2	1.20	0.276	0.23	YYD	Broad
2B10A2	ND	0.680	ND	YYD	Broad
2B10B4	1.65	0.856	0.52	YYD	Broad
2B10D7	1.29	0.737	0.57	YYD	Broad
2B10D5	1.15	0.761	0.66	YYD	Broad
2B10E8	1.02	1.12	1.1	YND	Narrow
2B10B10	1.99	3.39	1.7	YNY	Narrow
2B10E2	1.67	3.18	1.9	YNY	Narrow
D7	1.55	5.51	2.9	YNY	Narrow

The binding kinetics were determined for a sampling of VHH from the family specific library, and the VHH were ranked according to decreasing affinity (KD). The parental A12 and D7 took the pole ends of the table. VHH with a YYD102 motif have affinities of less than 1 nM, whereas VHH without a YYD102 motif have affinities of more than 1 nM, which is associated with a change in neutralization category from Broad to Narrow.

VHH Outputs Show Diverse Affinities

A selection of VHH from the family-specific library was tested in surface plasmon resonance to determine their affinity constants (K_D) with gp120 IIIB. The affinities of the VHH ranged from 0.1 to 2.9 nM, representing a 30-fold difference in affinities. When the VHH were ranked according to their affinities as shown in Table 5, VHH with higher affinities of < 1 nM possessed a YYD^{102} motif and invariably belong to the Broad neutralizer category, whereas VHH with lower affinities of > 1 nM are without the YYD102 motif and belong to the Narrow neutralizer category. Therefore, there is a clear association with the YYD^{102} motif for higher affinities to gp120 IIIB.

DISCUSSION

In this study, we have successfully used a novel method to create a family-specific library of HIV-1-neutralizing VHH containing variants that are homologous to the parental A12/D7 VHH that neutralize a broad spectrum of HIV-1 isolates (30). This was achieved through the use of specially designed primers that target the tail of the CDR3 sequence, thereby isolating sequence-homologous VHH from the diverse repertoire that have affinity matured in the immunized llama.

The CDR3 was targeted in this study as its importance in the classification of antibodies with similar epitope recognition was described for HIV-specific antibodies from human donors (53) and for VHH targeting bacteriophage proteins (41). The family-specific library was then panned on picomolar concentrations of recombinant gp120 in solution form to reduce potential avidity effects. As all the variants within the family were assumed to target the CD4bs of gp120 in a similar fashion to A12/D7, a general trypsin elution was employed to elute all bound phages. This method could elute out phages that might not be eluted by solubleCD4elution. This property of the family-specific library was confirmed with a competition ELISA experiment where all the isolated VHH tested were able to prevent soluble CD4 from binding to gp120. It is possible that post-translational modifications of VHH when expressed in mammalian cells might affect their properties, but this has not been studied to date. A high resolution crystal structure study of A12 bound to gp120 is currently being sought, which would help to elucidate the precise binding epitope of this VHH. Through this novel technique, we have isolated 49 unique VHH belonging to the A12/D7 family that had not been previously isolated when using the competitive elution method with soluble CD4. These variants were able to discriminate between two of the subtype C viruses tested, and these VHH were grouped according to their ability to neutralize the virus isolates tested. The molecular basis of this discrimination was mapped to the last two amino acid positions at the C terminus of the CDR3 loop. The YYD^{102} motif is correlated with the ability to neutralize both subtype C HIV-1 envelopes, C222 and 92BR025.C1. Any change in this motif was sufficient to abrogate the ability to neutralize either or both viruses, and this was confirmed

with mutational studies. Mutations in the other hot spot residues within the framework regions or CDRs did not affect this neutralization phenotype, although it might contribute to the wide range of IC50 values observed with C222 and 92BR025.C1. This would require further analysis to determine the contributory role of each residue involved in antigen specificity and binding to the various HIV-1 envelopes. A high resolution crystal structure of the D7 VHH has recently been solved and will help to shed some light on structure and function (54). There is also a direct correlation between the YYD^{102} motif and high binding affinities to gp120 IIIB, with changes within the motif resulting in decreased affinities. Antibody affinities in the high picomolar range are close to the suggested affinity ceiling for *in vivo* maturated antibodies (55, 56), which might explain the absence of VHH with even higher affinities than A12 in the original immune library. The diversity of HIV-1 viral envelopes restricts the generation of potent and broadly cross-reactive Nabs (57) and is a major obstacle for effective antibody-based immunization against HIV-1. The creation of such new antibodies is a high priority for HIV-1 vaccine development. This method can help to create a diverse panel of neutralizing antibodies that has the ability to recognize different virus isolates. The panel of VHH can be used for identification and characterization of conserved epitopes on HIV-1 envelope proteins that can serve as templates for the design of new immunogens, and also for potential use as therapeutics.

In conclusion, we have created a family-specific VHH library whereby members of the family show similarity in its ability to recognize a particular antigen. However, the diverse members of the family show variations in terms of their binding affinities, as well as neutralization ability across different strains of viruses. By analyzing the sequences of the VHH, we were able to associate certain amino acid residues as crucial to potent neutralization capacity and better recognition of a diverse set of HIV-1 Envs. Instead of the painstaking task of antibody engineering, the mining of a diverse pool of antibodies that has undergone *in vivo* affinity maturation has led to the creation of a panel of homologous antibodies, allowing the study of the

paratope that is essential for Env recognition and neutralization. These VHH may provide a basis for future engineering of antibodies against HIV-1 and

also offer new tools for development of inhibitory microbicides, vaccines, and research reagents.

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Chapter 3

Adapted from:

Antibody engineering reveals the important role of J segments in the production efficiency of llama single-domain antibodies in *S. cerevisiae*

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Variable domains of llama heavy-chain antibodies (VHH) are becoming a potent tool for a wide range of biotechnological and medical applications. Because of structural features typical of their single-domain nature, they are relatively easy to produce in lower eukaryotes, but it is not uncommon that some molecules have poor secretion efficiency. We therefore set out to study the production yield of VHH. We computationally identified five key residues that are crucial for folding and secretion, and we validated their importance with systematic site-directed mutations. The observation that all key residues were localized in the V segment, in proximity of the J segment of VHH, led us to study the importance of J segment in secretion efficiency. Intriguingly we found that the use of specific J segments in VHH could strongly influence the production yield. Sequence analysis and expression experiments strongly suggested that interactions with chaperones, especially with the J segment, are a crucial aspect of the production yield of VHH.

INTRODUCTION

In 1993 Hamers *et al.* discovered that members of the camelidae family possess not only conventional antibodies but also antibodies devoid of the light chain (Hamers-Casterman,C., Atarhouch,T. *et al.*, 1993). Variable domains of these heavy-chain antibodies (VHH) thus have only three complementarity determining regions (CDR) but they can nevertheless recognize their cognate antigens with binding constants equalling those of conventional immunoglobulins. Their high binding affinity and small size make them good candidates for applications ranging from biotechnology to medicine and their relatively large CDR3 may be an advantage in the recognition of epitopes in clefts or pockets unreachable by conventional antibodies (De Genst,E., Silence,K. *et al.*, 2006).

The importance of VHH as therapeutic agents is rapidly growing as they can be very specific and normally show little side effects (Borrebaeck,C.A. and Carlsson,R., 2001) in a wide variety of applications. The possibility to produce VHH as homo- or hetero-multimers (Conrath,K.E., Lauwereys,M. *et al.*, 2001), often with synergistic effects, further adds to their usefulness as therapeutic (Kruger,C., Hultberg,A. *et al.*, 2006, Saerens,D., Ghassabeh,G.H. *et al.*, 2008), anti-bacterial (Kruger,C., Hultberg,A. *et al.*, 2006), or anti-viral agents (Hultberg,A., Temperton,A.J. *et al.*, 2011, Jahnichen,S., Blanchetot,C. *et al.*, 2010).

We are developing VHH as microbicides to combat infectious diseases, in particular HIV (Forsman,A., Beirnaert,E. *et al.*, 2008)(Gorlani,A., Brouwers,J. *et al.*, 2011), in developing countries. To be successful, these microbicides should be affordable and physically stable under a wide range of conditions. We have developed continuous and fed-batch fermentation processes for VHH using *S. cerevisiae* as production host and often have reached high production yields (van de Laar,T., Visser,C. *et al.*, 2007). However, neither are all VHH equally well produced nor are they all equally stable. Despite almost twenty years of study into this topic, little is known to date about the production yield and stability (together called 'production efficiency') of VHH.

Production efficiency is a key factor that determines whether a promising molecule will become a marketable product.

It has been shown that VHH binding affinity is mainly determined by the CDRs while folding and secretion efficiency are mostly a function of the β-sheet scaffold (Nicaise,M., Valerio-Lepiniec,M. *et al.*, 2004). These characteristics allowed us to independently study and engineer the binding properties and the production efficiency (Saerens,D., Pellis,M. *et al.*, 2005). Obviously, the production efficiency of heterologous proteins is the sum of many factors. Previously we have studied several aspects of the production efficiency of VHH, including copy number of the heterologous gene, mRNA stability, presence of rare codons for *S. cerevisiae*, glycosylation, or charge and hydrophobicity of the protein surface (Lutje Hulsik,D.,2009). We here continue this systematic study with the identification of amino acids crucial for folding as folding efficiency has been shown important for production efficiency (Parekh,R., Forrester,K. *et al.*, 1995).

Vendruscolo *et al.* (Vendruscolo,M., Paci,E. *et al.*, 2001) studied folding using experimentally and computationally obtained Φ values (ratios of the change in stability of the transition state and native state associated with each amino acid) and found a limited number of residues with high Φ values. These residues made many contacts in the native structure *and* in the transition state of folding. They called them 'key residues' and concluded that the presence of their interactions in the transition state is crucial for proper folding. We have used this concept to computationally identify key residues in VHH, and we have experimentally validated their importance by means of site-directed mutagenesis.

The analysis of the location of the key residues in the 3D VHH structure led to the realisation that they are all in the vicinity of the J segment. As a consequence we analysed the combinatorics of variability (V), diversity (D), and joining (J) genes during the antibody maturation and found that the choice of J segment is important for VHH production yield in *S. cerevisiae*. As several J segments contain a chaperone BiP recognition signal, we also studied the relation between chaperones and production efficiency.

When expressed in yeast under the lead of the invertase signal peptide, VHH are co-translationally translocated to the endoplasmic reticulum (ER) and their folding occurs as the nascent polypeptide chain enters the ER (Ng,D.T., Brown,J.D. *et al.*, 1996). Protein folding is usually a faster reaction than translation/translocation (Fischer,G. and Schmid,F.X., 1990, Huard,F.P., Deane,C.M. *et al.*, 2006), unless interactions with chaperones are required to reach the native state, as is the case for immunoglobulins (Feige,M.J., Hendershot,L.M. *et al.*, 2010). If folding does not proceed optimally the protein will remain associated with ER-resident chaperone BiP (Brodsky,J.L., Werner,E.D. *et al.*, 1999). Extended association with BiP or other mechanisms (Werner,E.D., Brodsky,J.L. *et al.*, 1996, Meerovitch,K., Wing,S. *et al.*, 1998) can label the protein as a substrate for ER-associated degradation.

The ER is a complex system in which many chaperones, cofactors and synthesized proteins interact with each other (Braakman,I. and Bulleid,N.J., 2011). Despite similarities, the ER of lower eukaryotes like *S. cerevisiae* is less evolved than that of mammalian organisms where llama antibodies are naturally produced. Production yield differences observed in yeast as function of the J segment were not observed in Human Embryonic Kidney cells, supporting the hypothesis that interactions between J segment and chaperones are an important contributor to VHH production yield.

MATERIALS AND METHODS

Strains, media and culturing conditions

S. cerevisiae VWK18 gal1 (CEN-PK 102-3A, MATa, leu2-2, ura3, gal1::URA3, MAL-8, MAL3, SUC2) was used for all production efficiency tests. Preparation of all VHH constructs was carried out using *E. coli* strain TG1 supE hsd-5 thi$^-$ (lac-proAB) F'[traD36 proAB+ lacIq lacZ-M15]. The expression tests in mammalian cells were performed in Human Embryonic Kidney cells (HEK293E) by U-Protein Express BV, Utrecht, The Netherlands.

Selective medium for *S. cerevisiae* was Yeast Nitrogen Base (YNB) without amino acids, 6.7 g/L, glucose 20 g/L. Expression medium was YP broth (Yeast Extract, 10 g/L, Bacto Peptone, 20 g/L), glucose 20 g/L, galactose 5 g/L. LB medium, supplemented with ampicillin (100 μg/ml) was used for the selection of transformant *E. coli* colonies.

S. cerevisiae cultures were grown overnight at 30 °C,180 rpm, in selective medium, then diluted 1:100, to OD_{600} 0.03-0.08 in expression medium and incubated at 30 °C, 190 rpm for 48 hours. *E. coli* cultures were grown in selective LB medium (Bacto Tryptone, 10 g/L, Yeast Extract, 5 g/L, NaCl 10 g/L) overnight at 37 °C, 200 rpm.

Mutants construction

VHH R2 was used for the key residue experiments. All point mutations were introduced by QuickChange Site-directed Mutagenesis (Stratagene) following manufacturer's instructions. Primers of 25-27 nucleotides in total, with the mutated codon roughly in the middle of the sequence and flanked by 8-14 wild type nucleotides at each side were designed. Replacement of β-strand 9 was done by PCR. We used a forward primer that annealed to a region within the signal peptide at the 5' of the VHH gene, containing the restriction site PstI, and custom-designed reverse primers encoding the DNA sequence specific for J4- and J7- β-strand 9 and the BstEII restriction site for compatibility with

vector pUR4585. PCR products of the expected length were excised from agarose gel, digested and ligated in pUR4585.

After transformation of *E. coli* and confirmation by DNA sequencing, the purified plasmid DNA was transformed into *S. cerevisiae* using the lithium acetate method (Gietz,R.D. and Schiestl,R.H., 2007). At least 5 transformant colonies were screened for VHH secretion.

Protein analysis

10 µl of culture supernatant were mixed with sample buffer (80 mM Tris/HCl pH 6.8, 33% glycerol, 6.7% SDS, 300 mM DTT, 0.01% bromophenol blue), boiled for 10 minutes and loaded on 15% poly-acrylamide gels. After electrophoretic separation, proteins were blotted onto Immobilon membrane (Millipore, MA, USA). Detection of the VHH protein band was performed with Protein G-purified anti-VHH rabbit serum 1:5000 in milk/1% PBS for 1 hour. Secondary antibody Goat Anti-Rabbit (Li-Cor Biosciences, NE, USA) complexed with an infrared dye IR800 (1:10000 in milk/1% PBS for 1 hour) was used for detection of the rabbit serum.

Protein purification

*S. cerevisiae c*ultures were centrifuged in a Sorvall RC-5BPlus at 6000 rpm for 15 minutes to pellet cells. Fermentation medium was subsequently passed through 0.22 µm PES membrane filter for clarification after which we used the VivaFlow 200 (Sartorius AG, Germany) ultrafiltration device to reduce the volume and to exchange buffers.

Samples were affinity-purified using an Akta Xpress machine (GE Healthcare Europe, Diegem, Belgium) equipped with HisTrap FastFlow 1 ml column (GE Healthcare). Binding buffer was 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 20 mM Imidazole. Elution buffer was 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 500 mM Imidazole. Immediately after elution, VHH were desalted to PBS using 2x 5 ml HiTrap Desalting columns, (GE Healthcare). Purified proteins were stored at -20 °C.

CD spectra and T_m measurement

Jasco J-810 Spectropolarimeter (Jasco Inc., Great Dunmow, UK) was used to obtain CD measurements and T_m values of VHH. Purified VHH were dialyzed against MilliQ water prior loading the machine. After identifying 205 nm wavelength as optimal for following state changes in VHH, a temperature gradient 20 °C to 90 °C (unfolding) and back to 20 °C (refolding) was applied to the samples, with a slope 2°C/min.

Bioinformatic tools and analysis

WHAT IF (Vriend,G.,1990) was used for sequence and structure analyses and homology modelling. YASARA (*www.yasara.org*) was used for visual inspection of 3D structures. Llama VHH structures were collected from the Protein Data Bank (Berman,H.M., Westbrook,J. *et al.*, 2000). The following structures were used: 1G9E (Renisio,J.G., Perez,J. *et al.*, 2002), 1HCV (Spinelli,S., Frenken,L. *et al.*, 1996), 1I3U (Spinelli,S., Tegoni,M. *et al.*, 2001), 1I3V (Spinelli,S., Tegoni,M. *et al.*, 2001), 1QD0 (Spinelli,S., Frenken,L.G. *et al.*, 2000), 1SJX (Dolk,E., van der Vaart,M. *et al.*, 2005), 1U0Q (Cambillau, unpubl.), 2BSE (Spinelli,S., Desmyter,A. *et al.*, 2006), 2XA3 (Hinz,A., Lutje Hulsik,D. *et al.*, 2010) and 3EZJ (Korotkov,K.V., Pardon,E. *et al.*, 2009). 1152 unique llama VHH sequences, obtained from proprietary and public databases, were used in this study (http://swift.cmbi.ru.nl/mcsis/systems/ABVDDB/).

Determination of important residues for *in vitro* folding was done with WHAT IF's implementation of the criteria of Vendruscolo *et al.* For each residue the Cα distance to each other Cα was measured, excluding the direct neighbouring residues. Only residues that made five or more contacts in all structures were taken into account. The standard WHAT IF options for mutant prediction and analyses were used to analyse the structure of mutant VHH and to analyse their gain or loss of contacts.

To determine the most likely germline genes of V and J segments, an alignment using WHAT IF's implementation of all-against-all combined DNA-protein alignments were done. The DNA sequences were translated in the correct reading frame, resulting in the following sequence format:

cagQgtgVcagQ, i.e. first the codon, then the corresponding amino acid. Each sequence was stored using a special format, called a DNA-protein sequence, which dictates that the triplet and the corresponding amino acid remain associated and in-phase in subsequent alignment procedures.

RESULTS

Determination of key residues for folding and secretion *in vivo*

Vendruscolo *et al.* (Vendruscolo,M., Paci,E. *et al.*, 2001) identified and described, in the enzyme acylphosphatase, the interactions of a small number of amino acids that form a native-like contact network in the transition state, which was found to be essential to efficiently obtain the native fold of the protein. They called these residues 'key residues' and observed that they have more interactions with other amino acids, compared to non-key residues.

We analyzed ten 3D structures of VHH available from the PDB and we picked out between 28 and 40 amino acids in each structure that make an above-average number of contacts, like key residues, and thus are likely to form the points of nucleation for the folding of the protein. All their interactions take place with amino acids belonging to the VHH framework, and not to the CDRs. An alignment of these ten VHH sequences revealed that eight of the putative key residues were conserved: L20, W36, R38, V63, M82, L82c, D86 and Y90 (Kabat numbering). These same residue positions are also highly conserved in the non-redundant database of 1152 VHH sequences from immunized and non-immunized llama libraries that we utilized in this study.

Using VHH R2 (Spinelli,S., Frenken,L.G. *et al.*, 2000) as a model protein, we performed an alanine scan on the hydrophobic residues and made conservative mutations on the three hydrophilic ones. Additionally we introduced mutations that were suggested by the alignment of the 1152 VHH sequences. We constructed L20A, W36A, V63A, M82A, L82cA, D86A, Y90A and L20V, L20I, R38K, D86E, Y90F. The conservative mutations were meant to maintain properties of the wild type amino acid, as opposed to the introduction of alanine. Table 1 shows that the introduction of alanine at positions 20, 36, 38, 86 and 90 abolished secretion, whereas introduction of alanine at positions 63, 82 and 82c reduced the secretion to 20-70% of wild type VHH R2. Conservative mutations had in some cases mild effects on

Table 1. Effect of key residue mutations on secretion and on thermal stability.

Amino acid[a] (Kabat)	Number of interactions	Mutation	Residual secretion (WT = 100%)[b]	Δ T_m (T_m mutant - T_m wild type)[b]
L20	7	L20A	0%	n.d.[c]
		L20V	0%	n.d.
		L20I	40%	-14.2
W36	8	W36A	0%	n.d.
R38	5	R38K	30%	-42.8[d]
D86	5	D86A	0%	n.d.
		D86E	5%	-7.3
Y90	5	Y90A	0%	n.d.
		Y90F	25%	-1.3
V63	6	V63A	15%	-5.5
M82	5	M82A	20%	-5.9
L82c	5	L82cA	70%	-6.6

[a] The indicated amino acids were replaced by alanine or by amino acids similar to the wild type (conservative mutation). [b] Secretion in S. cerevisiae and melting temperature (T_m) were measured and compared to the wild type VHH. [c] Not determined because not enough material could be obtained. [d] Aggregates of the mutant VHH R38K started appearing at 30 °C, thus the measurement was stopped.

secretion, like L20I (40% of wild type), R38K (30%) and Y90F (25%), while in other cases strong effects were observed, like L20V (0%) and D86E (5%). On the base of the effect on secretion we concluded that only five of the eight putative key residues are crucial for secretion. Moreover, we observed a lower melting temperature compared to VHH R2 wild-type in all mutants for which

enough material could be obtained. We did not find a correlation between the loss in stability and the loss in production yield.

Analysis of VHH segments and their association with secretion in *S. cerevisiae*

Figure 3A and 3B show the location in the VHH structure of the 8 residues tested. It struck us that seven out of eight putative key residues are located in the vicinity of β-strand 9, the strand encoded by the J segment. The five crucial and two non-crucial key residues form a hydrophobic groove where the J segment docks upon folding. This prompted us to study the relation between V and J segments and the production yield.

We expressed in *S. cerevisiae* 71 VHH that were part of the 1152-sequence database. These 71 VHH were selected from different libraries, recognize different antigens, and the crucial key residues were present in all of them (except for VHH 7, 8, and 57, that carried the mild L20I substitution that was showed non crucial for production yield). We measured the amount of VHH present in the culture medium and defined five secretion classes, from low to high yield (Figure 1). Despite that it is commonly believed that VHH secrete well, we observed a 100-fold production yield difference from class 1 to 5 and almost half of the VHH measured had a secretion level <10 mg/L.

VHH originate from the joining of V, D, and J genes and concomitant and/or subsequent maturation of these genes. V genes encode for the largest part of the VHH, from framework 1 to 3 including CDR1 and CDR2. D genes encode for most of CDR3 and J genes for framework 4, also termed β-strand 9. We investigated whether certain of V, D, J combinations correlate with the secretion level in *S. cerevisiae*. We matched the 71 sequences with the 23 germline genes that encode for their V segment and linked the genes to the secretion class of the VHH. The same was done with the 7 germline genes encoding for the J segment. The D segment consists of essentially CDR3 and it is mainly involved in antigen recognition therefore it was not considered.

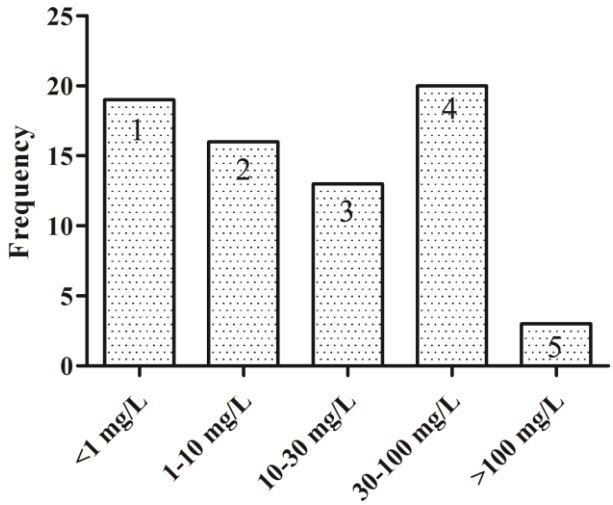

Figure 1. Ranking of the production yield of 71 VHH. Five classes were defined, from <1 mg/L to >100 mg/L of VHH secreted in culture medium. Almost half of the VHH measured had a secretion level <10 mg/L.

In the large sequence-database 23 V and 6 J genes were found, whereas 18 V and 3 J genes were represented in the 71 VHH that we expressed.

No relation was found between V germlines and secretion class, whereas it appeared that J genes 4 and 6 were the only genes represented in the two highest secretion classes, while J7 was almost exclusively found in the two lowest secretion classes (Table 2).

J segment (β-strand 9) swap between class 1 and class 5 VHH

To study the role of the J segments further, we analysed VHH3 and VHH70. These two VHH are in the highest and lowest secretion class, respectively, and they bind the same antigen. These VHH use the same V gene but use a J4 and J7 gene, respectively. Investigation of their modelled 3D structure showed that the inter β-strand interactions and the conformations of both β-strands 9 are very similar. We exchanged the J segments between the two VHH and measured the production yield. Mutant VHH70 (VHH70-J4) had a secretion

Table 2. Relation between V and J germline genes usage and secretion class of VHH.

Germline[a]	Secretion Class[b]		
	1/2	**3**	**4/5**
Vc-Ve	2	0	0
Vg-Vj	7	0	2
Vk-Vn	4	2	8
Vo-Vq	3	2	2
Vr-Vt	12	5	11
Vv-Vw	5	3	0
J4	18	8	17
J4 or J6	8	4	6
J7	9	1	0

[a] Four V and four J genes were not found in our dataset. [b] No significant correlation exists between V genes and secretion yield. J7 gene correlates with low secretion yield in S. cerevisiae (p= 0.01).

yield comparable to VHH3, while mutant VHH3 (VHH3-J7) was almost not secreted (Table III). In this experiment the J gene 7 seemed the bottleneck for high secretion.

VHH3 and VHH70 shared the same V gene. We therefore investigated whether the effect of the J gene would be independent from the V gene used. VHH A12 (Forsman,A., Beirnaert,E. *et al.*, 2008) and 3F3 (Strokappe,N.M., Szynol,A. *et al.*, submitted) fitted the requirements because they originated from different V genes, and their J genes were J4 and J7, respectively. A12 was naturally well-secreted whereas 3F3 was not. Swapping the J segments had on these molecules the same effect observed for VHH3 and VHH70 (Table 3), thus strengthening the suggestion that the J segment is an important factor in the production efficiency, independently from the V segment used.

Table 3. Characterization of VHH – J segment chimeric constructs.

Protein	J segment used	Secretion class	T_m (°C)	Refolding ability
VHH3 WT	J4	5	74.5	+
VHH3 mut	J7	1	70.1	-
VHH70 WT	J7	1	70.8	-
VHH70 mut	J4	5	76.7	+
VHH A12 WT	J4	5	74.2	+
VHH A12 mut	J7	1	n.d.	n.d.
VHH 3F3 WT	J7	1	n.d.	n.d.
VHH 3F3 mut	J4	4	73.9	+

Thermal stability of VHH3, VHH70, and J-segment mutants.

Figure 2 shows the thermal unfolding and the refolding profile of VHH. Thermal denaturation occurred for VHH3 at a higher temperature (74.5 °C) than VHH70 (70.8 °C). However, introduction of J4 in VHH70 increased its thermal stability by almost 6 degrees, to 76.7 °C and consistently, introduction of J7 in VHH3 had the opposite effect, lowering the unfolding temperature to 70.1 °C. The unfolding of VHH3 wild type and VHH70-J4 was reversible whereas VHH3-J7 and VHH70 wild type would not refold when the temperature decreased. It seems that the presence of J4 relates to both a higher T_m and to reversibility of folding. These data indicate an important role played by the J segment.

Expression of VHH3 and VHH70 in HEK293 cells.

Although protein stability and production yield often correlate (Kowalski,J.M., Parekh,R.N. *et al.*, 1998, Kwon,W.S., Da Silva,N.A. *et al.*, 1996), we observed that this relation does not hold very well when we mutate key residues that are involved in the proper association of β-strand 9 with the rest of the VHH framework. We therefore studied whether aspects of the cell folding

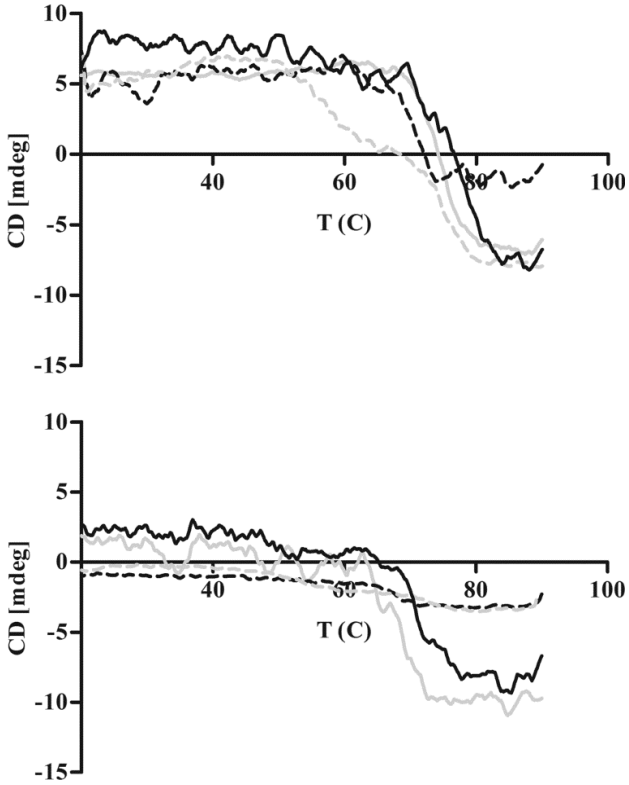

Figure 2. **Circular dichroism spectra of VHH.** Circular Dichroism of VHH was measured over a thermal gradient to determine their melting temperature. VHH3 wild-type: grey solid lines; VHH3-J7: grey dashed lines; VHH70 wild-type: black dashed lines; VHH70-J4: black solid lines. Thermal unfolding occurred at higher temperature when both VHH3 and VHH70 used J4 segment compared to J7. Refolding was not possible when VHH used J7 segment.

machinery could explain the observed effects by expressing a series of variants in HEK293 cells. These cells have a more developed system of chaperones than the yeast cells, and the difference between mammalian chaperone BiP and its yeast homologous Kar2p may be a decisive factor (Rose,M.D., Misra,L.M. *et al.*, 1989). Interactions with the nascent immunoglobulin chain serve as temporary stabilizer while the protein disulfide isomerase (PDI) assists the formation of the conserved cysteine bridge (Mayer,M., Kies,U. *et al.*, 2000). In the ER, BiP/Kar2p binds specific motifs of seven amino acids, consisting

of large hydrophobic residues alternated with small ones (Blond-Elguindi,S., Cwirla,S.E. *et al.*, 1993). Such a motif is found in β-strand 9 of VH and VHH domains (Knarr,G., Gething,M.J. *et al.*, 1995). Due to differences in the peptide-binding pocket, the Kar2p-VHH interactions might be less stabilizing than BiP-VHH in the presence of a J7-derived β-strand 9. We therefore tested whether J segments had an effect on the production of isolated VHH domains in mammalian cells. Results of the expression of VHH3 and VHH70 in HEK293 cells showed that the secretion difference between the two proteins was negligible. This supported our hypothesis that in *S. cerevisiae* the low production yield of VHH using J7-derived β-strand 9 is caused by suboptimal interactions between Kar2p and the folding VHH. In fact the 3D models of VHH3 and VHH70 revealed that the side chains of the solvent-accessible residues in β-strand 9 form different hydrophobic motifs, as highlighted in the dashed area recognized by BiP/Kar2p (Figure 3C). We recorded CD spectra of VHH produced in mammalian cells and we compared them with spectra of yeast-produced VHH that we previously acquired. We observed that both the unfolding and refolding transitions occurred in the same manner and at the same temperature, confirming that the native conformation of VHH is not dependent on the host used for their production.

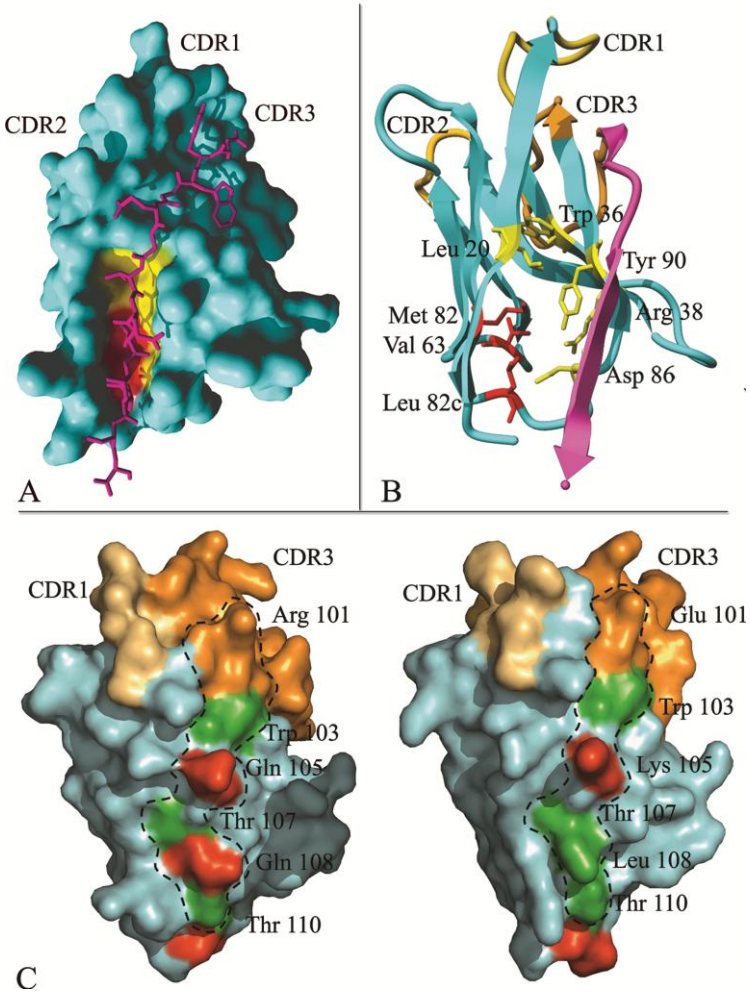

Figure 3. A) The surface of VHH R2 with β-strand 9 left out is shown in cyan. After surface calculation, β-strand 9 was put back as a purple stick model. The surface made up by the 5 key residues that essentially abolish production yield if mutated to alanine is shown in bright yellow, whereas the surface of residues M82 and L82c is in red. V63 does not contribute to this surface. B) 3D ribbon representation of VHH R2. The scaffold is shown in cyan and the three CDR loops are in shades of orange. Crucial key residues L20, W36, R38, D86 and Y90 are shown in yellow, residues V63, M82, L82c are in red. β-strand 9 is represented in purple. C) Molecular surface of VHH3 (left) and VHH70 (right). The scaffold is shown in cyan and the three CDR loops are in shades of orange. β-strand 9 residues are shown in green (hydrophobic side-chain) or red (hydrophilic side-chain). BiP-recognized motifs according to Blond-Elguindi *et al.* are circled with a dashed line. Note that β-strand 9 derived from J4 (left) and J7 (right) display different hydrophobic patterns.

CONCLUSIONS

The growing importance of llama heavy-chain antibody fragments, VHH, in health care and as research and imaging tools has raised the question how to produce them efficiently. We use *S. cerevisiae* as production host because of its outstanding record in biotechnology and because it is a 'generally recognized as safe' (GRAS) microorganism (Buckholz,R.G. and Gleeson,M.A., 1991, Gellissen,G., Melber,K. *et al.*, 1992, Idiris,A., Tohda,H. *et al.*, 2010).

We previously analyzed several host- and process-related factors (Lutje Hulsik,D.,2009) and here we looked at key residues and specific V or J genes as determinants of secretion. We found that amino acids L20, W36, R38, D86, and Y90 behave like Vendruscolo's key residues and we determined that they are very important for the proper folding of VHH *in vivo*. However key residues are not more important than other residues for the stability of the structure, as demonstrated by the comparable loss in T_m of key residue mutants and the other destabilizing mutants V63, M82 and L82c.

The observation that all five crucial key residues are located close to the side of the β-sandwich where β-strand 9 is meant to fold brought our attention to its role in folding. The large difference in secretion efficiency recorded among the 71 VHH tested, despite all of them having the five key residues in place, suggested an important role in folding and production yield for the β-strand 9 that is encoded by the J gene. The correlation of J gene 7 with inefficient secretion indicated that independently of the V segment, amino acids on the J segment can still have a strong effect on the folding. Once we identified J segments specific for high and for low production yield we were able to clone these J segments in other VHH (based on unrelated V and D segments) and transfer the beneficial or detrimental effects.

The involvement of chaperones in determining the folding efficiency of the J segment was confirmed by the parallel expression of a J4- and J7-related VHH in yeast and mammalian systems. Whereas *S. cerevisiae* has a rather primitive chaperone system, mammalian cells have excellent chaperones, and in fact the large secretion difference reported in yeast cells completely disappeared in mammalian cells. The equivalent thermal stability of yeast-produced and

mammalian-produced VHH is the proof that the folding pathway, rather than the final conformation, causes the production yield differences.

All observations together led us to postulate a two-step folding mechanism hypothesis for VHH. First, the N-terminal V segment folds and thereafter the C-terminal J segment docks on the groove formed on the V segment to complete the β-sandwich and to anchor the long flexible peptide constituted by the CDR3 and the J segment itself. It seems probable that chaperones are involved in protecting at least the J segment, but perhaps also the D segment, while the V segment folds.

Our work points out a new aspect of the production efficiency of VHH, which is the importance of folding kinetics, rather than the stability of the native structure. We anticipate that interesting results can be obtained by cloning mammalian chaperones such as BiP into the yeast used for VHH expression.

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Chapter 4

Adapted from:

Llama antibody fragments have good potential for application as HIV-1 topical microbicides

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There is an urgent global need for preventative strategies against HIV-1 infections. Llama heavy-chain antibody fragments (VHH) are a class of molecules recently described as potent cross-clade HIV-1 entry inhibitors. We studied the potential of a VHH-based microbicide in an application-oriented fashion. We show that VHH can be inexpensively produced in high amounts in the GRAS organism *S. cerevisiae*, resulting in very pure, and endotoxin free product. VHH are very stable under conditions they might encounter during transport, storage or use by women. We developed active formulations of VHH in aqueous gel and compressed and lyophilized tablets for controlled release from an intra vaginal device. The release profile of the VHH from e.g. a vaginal ring suggests sufficient bioavailability and protective concentration of the molecule at the mucosal site at the moment of the infection. The *ex vivo* penetration kinetics through human tissues show that the VHH diffuse into the mucosal layer and open the possibility to create a second defense layer either by blocking the HIV receptor binding sites or by blocking the receptors of immune cells in the mucosa. In conclusion, our data show that VHH have high potential for HIV-1 microbicide application because of their low production costs, their high stability and their favorable release and tissue penetration properties.

INTRODUCTION

With the HIV pandemic on the verge of entering its fourth decade, the need to develop effective and inexpensive preventative strategies remains an urgent global health priority[1]. Although there is promising progress in the development of a vaccine[2], there is also growing evidence that vaginally-administered HIV microbicides are effective in helping to reduce the incidence of sexually-transmitted infections, which account for the majority of new infections in the developing world[3, 4]. The recent positive outcome of the CAPRISA 004 trial has shown that it is possible to achieve a certain level of protection with a coitally-dependent vaginal gel[5]. Most HIV microbicide candidates currently being evaluated in clinical studies are either non-specific polymeric compounds formulated as vaginal gels and designed to prevent entry of the virus into host cells[6-8] or potent small-molecule antiretroviral compounds which inhibit the viral replication cycle[9-11]. However, the lack of specificity associated with polymeric microbicide candidates, coupled with clinical data suggesting a modest degree of protection at best[12], and the concern over the potential for development of resistant strains with long-term use of a single antiretroviral agent has forced the field to consider alternative classes of microbicidal compounds. A number of microbicide candidates are currently being evaluated for their potential as HIV inhibitors, including human antibodies b12[13] and a triple mix of monoclonal antibodies 2F5, 4E10 and 2G12.While initial efficacy data in non-human primates has been encouraging, the high costs associated with production/synthesis, difficulties in developing stable formulations, and poor tissue permeability are major obstacles that need to be overcome in order to progress these biomolecular approaches as practical microbicidal strategies[14].

In addition to conventional immunoglobulins, *Camelidae* family possesses a class of antibodies devoid of the light chain, named heavy-chain antibodies[15]. The variable domain of these antibodies (VHH) can be selected against virtually any target molecule[16-18], cloned and efficiently produced by relatively easy and inexpensive methods[19-21]. Previously, phage-displayed libraries of VHH isolated from llamas immunized with the glycoprotein gp120 from different HIV-1 clades (A, B´/C and C) have been constructed. These libraries

have been screened and neutralizing VHH have been selected against a panel of viruses from clades A, B and C[22], with some showing remarkable neutralizing activity and broad cross-clade recognition. The screening for clones with improved neutralization characteristics, targeting new epitopes and the construction of multimeric and chimeric molecules continues and we now have a panel of more than 60 VHH with specific and characterized activity against different epitopes on gp120 and gp41[23, 24]. Moreover we are characterizing new families of VHH against the cell receptors CD4, CCR5 and DC-SIGN that, together with the recent discovery of CXCR4 binders[25] open up the possibility of a double defensive layer based on virus-binding and cell receptors-binding. In this study, several important aspects that determine whether this class of molecules fulfills the requirements for topical HIV microbicides have been evaluated for the first time, including production, purification, thermal and pH stability, formulations and mucosal permeability.

METHODS

Production of VHH constructs by cell culture methods

Strains, vectors and cultivation media

Preparation of all the VHH constructs was carried out using *Escherichia coli* strain TG1 supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZΔM15]. *S. cerevisiae* strain VWk18 *gal1* (CEN-PK102-3A, MATa, *leu2-3, ura3, gal1::URA3, MAL-8, MAL3, SUC3*) was used for the fermentations. Inocula, fermentation media and growth conditions were the same as previously described[21]. Plasmids pUR4547 and pUR4585 (Bio Affinity Company (Leiden, The Netherlands) were used as expression vectors for VHH genes in *S. cerevisiae*. pUR4547 is a 2 μm-based *LEU2*-marked plasmid suitable for *GAL7* promoter-controlled expression of VHH fused to the yeast invertase signal peptide. pUR4585 is identical to it but encodes C-terminal myc- and his-tag[26].

Fermentations

Bioreactor Bioflo III (New Brunswick Scientific, NJ, USA) was connected via an AFS Biocommand Interface to a personal computer, and data such as pH, temperature, agitation and DOT (dissolved oxygen tension) were collected in real-time. Exhaust ethanol was analyzed with a Xendos 2500 infrared analyzer (both instruments from Servomex, Zoetermeer, the Netherlands). Samples were taken and optical density (OD) at 600 nm was measured to determine the amount of biomass in the vessel. In addition, 1 ml samples from the culture were collected in pre-weighed Eppendorf tubes and cells were pelleted via centrifugation. Subsequently, cells were washed with 1 ml water and dried at 100°C for at least 24 h. The weight of the dried cells was calculated and used as dry-weight data.

Downstream processing and protein purification

Following fermentation, cultures were harvested and the supernatant separated from the biomass by centrifugation (Sorvall RC-5B plus centrifuge, 5000 rpm,

15 min) and subsequently filtered (0.22 μm, Millipore, Billerica, MA, USA). Sample volume was reduced by tangential flow ultrafiltration (5000 MWCO Vivaflow membrane, Vivascience-Sartorius, Goettingen, Germany). VHH were captured and purified from the supernatant with an AKTA Xpress chromatographic system (GE Healthcare, UK). His-tagged proteins were affinity purified with a His-Trap 1 ml column (GE Healthcare, UK). Untagged proteins were purified by affinity chromatography using protein A (MabSelect SuRe, 5 ml column). Binding and elution buffers, specific for each column type, were the same as indicated by the manufacturer. For desalting, 2x HiTrap 5 ml, or 1x HiPrep 26/10 desalting column (GE Healthcare, UK) were used. The proteins were eluted in PBS pH 7.0 and stored at -20°C.

Enzyme Linked Immuno Sorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) were used to test activity of VHH following formulations, stress treatment and in vitro release tests. MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl rgp140 ZM96, rgp140 UG37 or gp120 IIIB [4mg/L]. Plates were blocked (200 μl 4% skimmed milk in PBS) and washed three times with PBST. VHH in 50 μl of milk 1%/PBS were added in 4-fold serial dilutions ranging from 40 to 0.01 mg/L, and incubated 1 hr at room temperature. After washing with PBST, detection was carried out with 50 μl anti-llama heavy-chain rabbit serum 1:2000 for 1 hr at room temperature and washed three times in PBS. Subsequently, plates were incubated with 50 μl peroxidase-conjugated goat anti-rabbit monoclonal antibody 1:5000 for 1 hr at room temperature. Peroxidase activity was developed by adding 50 μl *o*-phenylenediamine in the presence of H_2O_2. The reaction was stopped after 30 mins with 25 μl H_2SO_4 1M. Absorptions at 490 nm were measured using a microtiter plate reader.

Preparation and characterization of VHH A12 gel formulations

An aqueous solution comprising sorbic acid (0.1% w/w), citric acid monohydrate (1.05% w/w), sodium citrate dihydrate (1.48% w/w) was prepared and adjusted to pH 4.5. Lyophilized VHH A12 powder (0.1-1% w/w) was added to the solution followed by the gelling agent hydroxyethyl cellulose (HEC, Natrosol 250 HHX, 1.5% w/w). Clear homogeneous gels were formed upon mixing. In addition to these citrate-buffered gels, unbuffered gels were prepared by replacing citric acid and sodium citrate in the formulation with NaCl (0.85%) to maintain osmolality. The gels were characterized with respect to pH, osmolality (The Advanced Osmometer 3250, Advanced Instruments, Norwood, MS) and viscosity (37°C; SV-10 vibrational viscometer, A&D Company, Tokyo, Japan). VHH precipitation in gel formulations (0, 1, 5 or 10 mg/g VHH) was investigated by optical density measurement (OD_{600}, Tecan Infinite M200 microplate reader, Tecan Benelux, Mechelen, Belgium) 60 min after dilution of the gels (50 µl) with vaginal fluid simulant (VFS, 20 µl) and semen simulant (SS, 70 µl). These volumes correspond to the application of 2.5 ml of gel (a typical dose volume in man) in 1 ml of vaginal fluid and 3.5 ml of seminal fluid[27, 28].

Manufacture and characterization of vaginal rings containing VHH A12

Silicone elastomer (MED-6382, Nusil Technology, Carpinteria, US) vaginal ring devices, having 54 mm overall diameter, 7.6 mm cross-sectional diameter and comprising three open-ended 3.0 mm diameter channels for insertion of solid dosage forms (tablets) of appropriate dimensions, were prepared by reaction injection molding (80°C, 2 minutes) using a laboratory-scale ring-making machine fitted with stainless steel ring injection molds. Solid dosage inserts containing VHH A12 were prepared by two different methods: direct compression of a powdered mixture of VHH and hydroxypropylmethylcellulose (HPMC) and lyophilisation of an aqueous hydroxypropyl methylcellulose (HPMC) gel containing VHH A12. For the directly compressed tablet inserts, 50.0 mg of lyophilized VHH A12 was mixed with 950.0 mg of HPMC (100,000 cps, Sigma Aldrich, Dorset, England)

in a Speed Mixer DAC 15FVZ-K (Synergy Devices Ltd.) for 1 min at 3500 rpm. Approximately 75.0 mg quantities of this active mix were compressed using a single punch tablet press (Riva Minipress) fitted with tablet die (3.2 mm deep, 3.0 mm wide and 7.6 mm long). A single insert was then located within each of two channels within the vaginal ring device. For the lyophilized inserts, 20.0 mg of lyophilized VHH A12 and 180.0 mg of HPMC were added to 1.8 ml of HPLC grade water and mixed at 3500 rpm for 1 minute in the SpeedMixer, before allowing hydrating overnight at 4°C to form a clear homogeneous gel. The gel was then injected into silicone elastomer tubing (SFM3-3650, SF Medical, 3.3 mm internal diameter, 6.35 mm outer diameter). The tubing was cut into 2.5 cm lengths and subsequently lyophilized (protocol described in table 1). The lyophilized rods were removed from the silicone tubing, cut to length and inserted into the channels of the vaginal ring device. Four such rings were prepared, each containing two VHH-loaded lyophilized inserts.

Individual inserts (n=4) and vaginal rings with inserts (n = 4 for each formulation) were individually placed into sealed flasks containing 10.0 ml and 30.0 ml of HPLC grade water, respectively. Each flask was placed in a temperature-controlled orbital shaking incubator (Unitron Infors, 37°C, 60 rpm, throw diameter 2.5 cm). The release medium was sampled daily for seven days with complete replacement of the release medium. Quantitation of VHH release was performed by SEC-HPLC with UV detection (PerkinElmer series 200 HPLC system) and employing a 50 mM ammonium acetate mobile phase adjusted to pH 5.5.

Table 1. Freeze drying protocol.

Freeze drying steps	Temperature (°C)	Time (min)	Vacuum (mTorr)	Condition
Cooling cycle	-28	240	100	Hold
Primary drying cycle	-20	60	100	Ramp
	-20	120	100	Hold
	-0	120	100	Ramp
	-0	120	500	Hold
	+20	120	500	Ramp
	+20	240	50	Hold
Secondary cycle	+27			
Post-heat settings	+20	1000	50	

Permeability of VHH through human vaginal tissue

Healthy human vaginal tissue was obtained from five postmenopausal patients, ages 49-70 years (mean age 59 ± 9 y SD) following vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa.

No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the different specimens. The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study. Specimens were treated and stored as previously described[29, 30]. VHH labelled with fluorescein isothiocyanate (FITC) were prepared according to the vendor's instructions (Molecular Probes, Inc., Eugene, US). The tissue specimens were carefully cut, into sections (4 mm diameter) and mounted in flow-through diffusion cells (exposed areas 0.039 cm^2) with the epithelial surfaces facing upwards. The tissue disks were then equilibrated for 10 min with PBS (pH 7.4) at 37 °C in both the donor and acceptor compartments of the diffusion cells. The PBS was removed from the donor compartment and replaced with 1.0 ml of 0.5 mM solution of FITC-labeled VHH in PBS. PBS at 37 °C was pumped through the acceptor cell at a rate of 1.5 ml/h and collected, by means of a fraction collector, at 2h intervals for 24 h. FITC-labeled VHH was detected using a fluorometric method (520 nm emission and 497 nm excitation, Perkin-

Elmer spectrophotometer, Perkin-Elmer, MA, USA). Permeation studies were performed on seven tissue replicates for each patient. Additional permeation studies were performed using de-epithelialised tissue specimens; de-epithelialisation was achieved by submerging the thawed vaginal tissue in water at 80 °C for 30 s, followed by removal of the epithelial layer with tweezers. For all experiments, flux (J) values of FITC-labelled VHH across the vaginal membrane were calculated by means of the relationship $J = Q/A.t$, where Q = quantity of compound crossing membrane (pmol), A = membrane area exposed (cm^2) and t = time of exposure (min). Steady state permeation was assumed for a particular vaginal mucosal specimen and VHH when no statistically significant differences ($p<0.05$, ANOVA and Duncan's multiple range test) between flux values were obtained over at least two consecutive time intervals.

RESULTS

Production of VHH by fermentation

After selection from phage libraries and characterization of specific activity, genes encoding for neutralizing VHH[22, 24] were cloned into vectors pUR4547 and pUR4585 and subsequently transformed into *S. cerevisiae*. Transformant cells were screened and the colonies with the highest secretion level were selected and used for fermentative production (data not shown). Feed rate was set to a fixed value: 1 liter feed per 12 hours. Taking into account the constant increase in volume due to addition of medium, dilution rate at t=0 h was 0.055 h^{-1}, at t=6 h was 0.041 h^{-1} and at t=12 h was 0.033 h^{-1}. This was close to optimal dilution rate of 0.06 h^{-1} described previously for VHH production[21]. After 12 hr of feeding, 1 liter fermentation fluid was harvested from the vessel in sterile conditions; then a second round of feed was started when the culture parameters stabilized. Three rounds of feed-harvest were performed each fermentation run.

Two types of fermentations were carried out: one based on glucose as carbon source, and the other one based on ethanol. We previously recorded a lower yield of purified protein in the fed-batch process based on glucose as sole carbon source compared to the ethanol-based fermentation (58.9 mg/l vs. 85.6 mg/L). Therefore, ethanol was selected as the preferred carbon source for the feed phase, while the batch phase employed glucose. In ethanol-fed fermentations we obtained biomass yields up to 71 ±11 g biomass (dry weight) per liter of culture. The yield of purified product obtained ranged between 90.7 and 109.6 mg/L of fermentation fluids.

Down-stream processing and purification

At the end of each run, recovery of secreted VHH from approximately 4.5 L of fermentation fluids included four steps. Cells were pelleted by centrifugation, the supernatant was filtered (0.22 μm pores membrane), and an ultrafiltration device (2x Vivaflow 200 membranes, MWCO 5Kd) was used to

reduce the volume to a few hundred ml. The processed supernatant was then loaded on a His-Trap column (GE Healthcare, UK) several times. High purity was obtained with all VHH tested with essentially no loss in the unbound fraction.

As an alternative to tagged proteins, VHH lacking tags were produced and purified using a protein A column (also from GE Healthcare). Protein A purification proved efficient and highly selective for these VHH. Protein purity in the eluted fraction was >95%.

Formulations

Gel formulation

A semi-solid gel formulation for vaginal administration of VHH A12 was developed and characterized. Preliminary experiments in which various vehicles were tested, demonstrated increased solubility of A12 (pI = 7.96) at acidic pH and relatively high ionic strengths. Based on these observations, a citrate-buffered vehicle (pH 4.5) with an ionic strength of 278 mM was selected for formulation of VHH A12 up to concentrations of 10 mg/g. Sorbic acid and the non-ionic polymer HEC were included as preservative and viscosity-enhancer, respectively. The basic characteristics of the gels were assessed in three independent batches: pH 4.6 ± 0.1, osmolality 253 ± 3 mOsm/kg and viscosity 1.34 ± 0.09 Pa·s. The binding activities of formulated (1 mg/g) and native VHH A12 were similar: the activity ratio formulated/native amounted to 0.97 ± 0.22 and it was consistent for the three clades used in ELISA. Upon storage of the gels for 2 months at 4°C, the ratio amounted to 0.93 ± 0.11; storage at 40°C, however, resulted in a decrease of activity to 0.56 ± 0.05 compared to freshly prepared gels (n=3).

In clinical use, gels will ultimately be diluted by vaginal fluid and, upon coitus, semen. Due to changes in the chemical environment, this dilution may alter the availability of VHH. In order to simulate this phenomenon, the gels (both buffered and unbuffered, 0-10 mg/g) were diluted with biorelevant volumes of

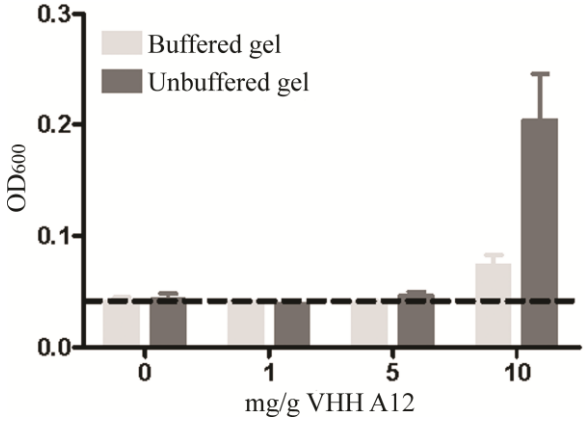

Figure 1. Increase in optical density (600 nm) upon dilution of gels with biorelevant media as a function of VHH A12 concentration. Buffered and unbuffered gels (50 µl) were diluted with 20 µl vaginal fluid simulant (VFS) and 70 µl semen simulant (SS). OD_{600} was measured after 60 min. The dashed line indicates the background signal (measured in absence of A12). Data represent mean ± sd, n=3.

vaginal fluid simulant (VFS) and semen simulant (SS). After 60 min, the optical density at 600 nm (OD600) was determined as a measure of possible precipitation of the VHH. Upon dilution with VFS alone, no precipitation was observed (data not shown). Similarly, dilution with both VFS and SS did not cause precipitation in gel formulations containing A12 at concentrations up to 5 mg/g. The high-dose gels (10 mg A12/g), however, showed an increased OD600, suggesting precipitation of VHH A12 (Fig. 1) in presence of VFS and SS**.** The increase in OD_{600} was about 4-fold higher for the unbuffered high-dose gel compared to the buffered one. The observed precipitation may be related to decreased solubility of VHH A12 in the relatively high pH of SS: in fact upon dilution with SS, pH increased from 4.6 to 7.7 in the unbuffered gels, but only from 4.6 to 5.8 in the buffered gels.

Vaginal Ring Formulations

Sustained release of VHH A12 was achieved over seven days for both directly compressed and lyophilized tablets inserted within a vaginal ring device. For the system having two lyophilized tablets inserted into the ring body, the total

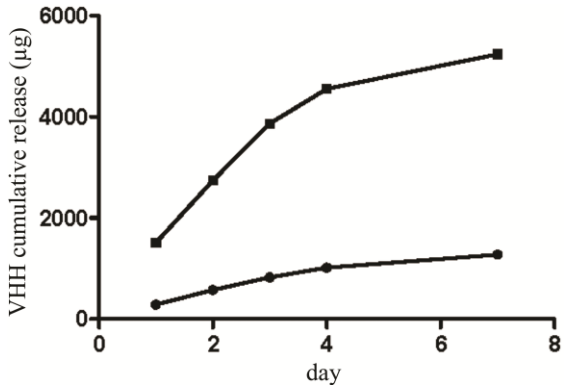

Figure 2. Mean *in vitro* cumulative release of VHH A12 from a vaginal ring device containing two compressed tablet inserts each loaded with 5% w/w VHH (square) and from a vaginal ring device containing two freeze-dried tablet inserts each loaded with 10% w/w VHH (circle). Cumulative release was measured at day 1, 2, 3, 4 and 7.

quantity of VHH released over 24 h and seven days was 289 and 1276 µg, respectively, equivalent to 17% and 76% of the initial VHH loading within the ring. By comparison, a single lyophilized tablet placed directly into the release medium (i.e not inserted into a ring body) provided in vitro release of 626 µg (75%) over 24 h. These results demonstrate that reduced surface area exposure of the tablet insert within the ring significantly reduces the daily release rate and extends the duration of release of the VHH. The mean cumulative release versus time profile observed for the ring devices comprising two directly compressed tablet inserts (5% w/w VHH loading per tablet) was very similar to that observed for the lyophilized tablet insert device except that the amount of VHH released was significantly greater (5.2 mg over seven days, equivalent to 67% of the initial VHH loading in the ring), a consequence of the higher absolute VHH loadings in these tablet inserts (Fig. 2). A single directly compressed VHH tablet placed in the release medium achieved 54% release over 24 h, although, unlike the lyophilized system, the tablet had not completely dissolved within this period. Binding data show that A12 formulated in the compressed tablet, inserted in the silicone holder preserves higher activity than when it is included in lyophilized tablets (99% vs. 92% activity respectively, compared to A12 control). The activity of VHH A12

released after 7 days in both the compressed and lyophilized tablet insert experiments was higher than 90% of the A12 control on all the clades tested.

Stability

Exposure to high temperatures due to lack of cold storage facilities and acidic pH in the cervicovaginal environment are important factors to be considered in the development of HIV microbicide products. Therefore, the ability of VHH to withstand thermal and pH stress was investigated. 1 mg/mL solutions of VHH A12 in PBS pre-incubated at 60°C, 70°C or 80°C for 10 minutes were more than 88% active compared to an untreated control (Fig. 3A). The thermal denaturation of VHH A12 and that of 4 other recently selected α-HIV VHH were compared and they were very similar, indicating that the remarkable heat tolerance is not unique to VHH A12[24].

Incubation of VHH A12 solutions in PBS at various pH (3.0, 4.0, 5.0 and 6.0; vaginal pH is <4.5[31]) for 48 h at 4°C demonstrated at least 87% of the original binding activity (Fig. 3A). In comparison, loss of binding was observed in the conventional antibody b12 starting from 24 h incubation at all tested pH. Moreover, after one week the activity of A12 and D7 was substantially unaltered (<10% loss) at any pH, whereas incubation at pH 4.0, 5.0 and even 6.0 was detrimental for b12 (1% of residual activity) and incubation at pH 3.0 caused a complete loss of signal in ELISA (Fig. 3B).

Permeability

Since HIV primary target cells (Langerhans cells and $CD4^+$ T cells) are localized in the vaginal mucosa[32], it is of critical importance for an entry inhibitor microbicide based on virus-binding as well as on cell receptors-binding, to be present in the tissues where the infection may occur. We investigated the diffusion kinetics of FITC-labeled VHH through human intact and de-epithelialised vaginal mucosa. De-epithelialised mucosa mimics the situation where the integrity of vaginal epithelium has been compromised,

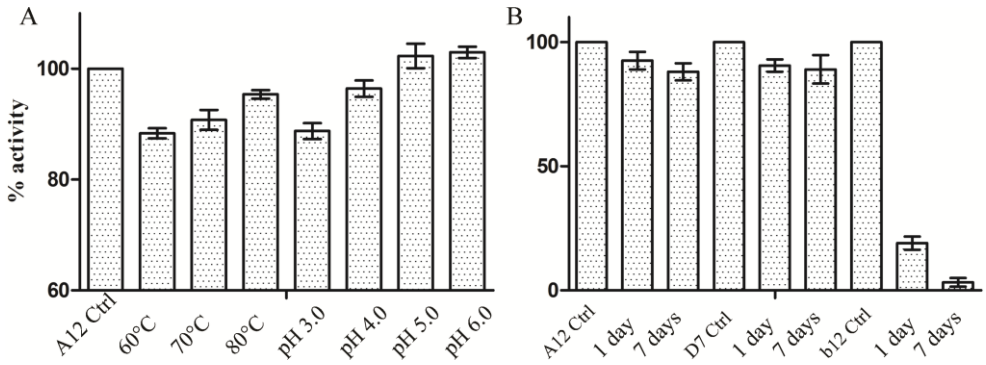

Figure 3. A) Activity of VHH A12 subjected to different pH conditions and temperatures. Incubation at pH 3.0, 4.0, 5.0 and 6.0 was performed at 4°C for 24 hours. VHH A12 was heated at 60, 70 and 80°C for 10 minutes in a water bath. Activity is expressed in percent compared to untreated control. VHH A12 retains at least 87% of original binding activity after treatment with low pH (pH 3.0) and at least 88% after treatment with high temperature (60°C). B) Activity of VHH A12, D7 and Mab b12 subjected to low pH (pH 3.0 to pH 6.0) for one day and one week at 4°C. Since the treatment with pH 3.0, 4.0, 5.0 and 6.0 had very similar effects, each bar summarizes the residual activities at the indicated time point for all pH treatments. While both VHH preserved at least 90% of their activity compared to untreated controls at both time points, incubation of b12 for one day caused a 10-folds reduction in activity, independently of the pH used. Incubation of b12 for one week at pH 4.0, 5.0 or 6.0 caused a 100-folds reduction in activity. Incubation of b12 for one week at pH 3.0 completely inactivated the antibody.

due to ulcerations associated with a variety of pathological conditions or sexual intercourse, even consensual. The antibody fragment diffused through intact and de-epithelialised vaginal mucosa at 37°C reaching steady state flux values after 4h of 10.24 ± 1.70 and 9.90 ± 1.74 $pmol.cm^{-2}.min^{-1}$, respectively. Contrarily to what we expected, the difference in permeability was quite small, and likely related to the removal of negatively charged epithelium. VHH A12 acquires a net positive charge at the physiological pH conditions under which the permeability experiments were conducted. The cationic antibody fragment will therefore be attracted to negatively charged epithelium, facilitating its partitioning from the aqueous buffer into the barrier domains of the intact mucosal membrane. De-epithelialisation, with the consequent loss of negative

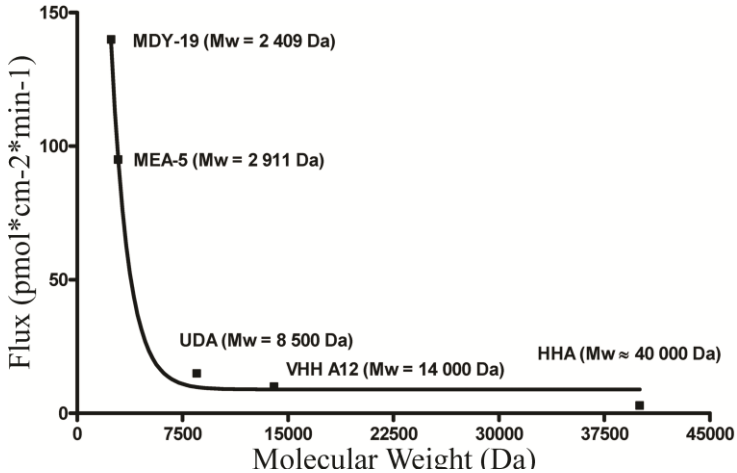

Figure 4. Mean estimated steady state (20-24h) values for five peptides/proteins across frozen/thawed human vaginal mucosa vs. molecular weight. VHH A12 flux fits in the model curve at its actual Mw value.

charge, decreases the attractive forces between the positively charged VHH A12 and the mucosa, thereby lowering its penetration potential into the mucosa. Fig. 4 shows the mean estimated steady state (20-24 h) flux values for five peptides/proteins across frozen/thawed human vaginal mucosa vs. molecular weight.

DISCUSSION

In this study we assessed the feasibility of a HIV-1 microbicide based on llama heavy-chain antibody fragments (VHH) capable of cross-clade neutralization. A few clones including A12 and D7 were previously characterized with a high potency of neutralization over a broad spectrum of HIV-1 isolates[22] and the structure of D7 and its mode of interaction with gp120 were described[33]. Currently our panel of VHH has increased to about 60 clones recognizing several epitopes on both gp41 and gp120 with some clones displaying breadth of neutralization better than the human monoclonal antibody b12[24].

A number of relevant properties for developing VHH as HIV microbicides are independent of molecular affinity of a specific clone, and can be associated more generally with the wider class of llama antibody fragments. An effective HIV microbicide will be impractical if the cost per dose is not affordable. We have developed methods for producing VHH using fed-batch fermentations based on ethanol as sole carbon source. Fed batch processes are routinely used by the biotech industry for production of bio-pharmaceuticals in large scale reactors up to 100 m^3[34]. Using a bioreactor with working volume of 2 liters we could affinity-purify about 100 mg of VHH per liter of fermentation fluids, and this yield can be increased by at least a factor 10 in industrial settings, as it was achieved in the production of anti-rotavirus VHH that is currently tested in a feeding trial of children in India (L. Hammarstrom, Personal Communication).

Several formulation approaches are being actively evaluated for vaginal administration of HIV microbicides, including gels, films and rings[35]. The preferred choice of formulation will ultimately depend upon many factors. A preferred formulation strategy would involve either once-a-day administration of a microbicide product (e.g. through use of a muco-adhesive, site-retentive gel formulation), or sustained release from a single vaginally-administered device over a period of many days or weeks. The feasibility of formulating VHH in a gel was evaluated. VHH A12 was formulated in a citrate-buffered gel with pH and osmolality falling within the physiological range of vaginal fluids, at concentrations up to 10 mg/g without losing binding activity.

Considering the use of microbicides in tropical climates, the observed drop in binding activity of VHH A12 upon storage of the gel formulation at elevated temperature (40 °C) requires further investigation. VHH were also formulated as solid tablets, using two different methods of manufacture (i.e direct compression and lyophilization), and inserted into a vaginal ring device for prolonged retention within the vaginal cavity. It is important to note that although the *in vitro* release results presented here specifically relate to a ring comprising two VHH-loaded tablet inserts, there is scope to produce ring devices with greater number of inserts, resulting in a proportionate increase in the amount of VHH released. Binding assays performed on VHH after gel incubation and tablet formulation showed that these molecules retain more than 90% of their original activity. Subjecting VHH to temperatures up to 80 °C and to acidic conditions (pH 3.0) caused activity to retain 88% and 87% respectively. More than 75% of original activity was also maintained after incubating VHH for a month at 37 °C. Taken together, these data show that VHH are robust molecules able to maintain high activity level in the harsh conditions met during storage and use as vaginal microbicide. No other conventional, full-chain immunoglobulin was so far shown to withstand such conditions.

Studies describing the correlation between *in vitro* and *in vivo* activity of the monoclonal antibody b12 have indicated that effective concentrations *in vivo* are typically up to 10^3-fold higher than *in vitro*[36] and the difference is even more accentuated in case of other microbicide candidates[37]. Assuming a vaginal fluid volume of 1 mL, the application of a single dose of 2.5 g gel containing 10 mg A12/g would result in vaginal concentrations that are about 10^3 to $2x10^6$-fold higher than the IC_{50} of VHH A12 measured on several tier 1 clade C viruses[22]. Given an average vaginal fluid production of 6 mL per day, the concentration of VHH A12 released every day over one week by compressed tablets inserted in a vaginal ring would be about $2x10^2$ to $3x10^5$–folds higher than the IC_{50} of A12 *in vitro* against some viruses. These data are promising and, provided VHH are selected against viruses used in current animal models, suggest that low doses are sufficient for full protection.

VHH A12 readily traversed both intact and damaged vaginal tissue at rates that are similar to those of similarly-sized molecules. This suggests that the vaginal epithelium does not work as such a tight barrier for the small antibody fragments. So VHH permeate into the sub mucosal tissue, where interstitial dendritic cells and Langerhans cells reside. The ability to traverse vaginal mucosa and enter the systemic circulation offers the possibility to create a second barrier either by blocking the receptor binding sites on the virus or by blocking the relevant receptors of the immune cells present in the vaginal mucosa. However the presence of VHH raises the issue of immunogenicity. Even though there is evidence that VHH do not elicit immune response nor create adverse effects because of their clearance via the kidneys, further studies need to be performed[38, 39].

In conclusion, we suggest that a coitally-related application of gel loaded with VHH or the formulation of VHH in IVR have the potential to perform as well as the successful 1% Tenofovir gel formulation in terms of drug thermal and proteolytical stability, bioavailability and cost-per-dose. A number of VHH resulted cross reactive for the i*n vitro* neutralization of SHIV (unpublished results) therefore suggesting that primate models can be readily used to test effectiveness of these molecules *in vivo*.

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General Conclusions

The development of preventative strategies to contain the HIV pandemic is looked at with great interest especially after recent success of the large trials CAPRISA 004[1] and iPrEX[2]. These trials demonstrated that a gel or pill formulations of molecules originally employed in the treatment of HIV-1 are effective at preventing sexually transmitted HIV-1 infections, provided they are used in the correct way and adherence is high. User friendliness, adherence, costs of manufacturing, distribution strategies, population behavior, are dominant factors determining the outcome of a HIV-preventative program[3].

Based on their physical properties and the potential of relatively cheap production and purification processes, llama single-domain antibodies (VHH) are promising molecules to serve as active components in microbicides. Therefore it was investigated whether neutralizing antibodies could be raised in *Lama glama*. Two different families of VHH were found that neutralized about 40% of the tested HIV strains[4].

We determined the 3D structure of one of these VHH in an attempt to better understand the mechanism of neutralization and to find ways to improve binding properties *in vitro*. Simultaneously, in our group other immunizations were carried out with new immunogens (gp140 from clades A and C, common in sub-Saharan Africa, and proteoliposomes, to reproduce the membrane-proximal environment). It was possible to select VHH against gp41 (L. Rutten, personal communication) and gp140[5] with promising neutralizing properties. Currently, we have 4 groups of VHH that recognize (partially overlapping) epitopes on the CD4bs and one group recognizing the MPER on gp41.

Two of the selected VHH, notably D7 and 1B5, were the object of mutational studies that had the goal of improving our understanding of antibody-antigen interactions and to enhance their properties. Cheap production of VHH is an absolute requirement for the development of microbicides. Therefore we investigated why certain VHH are produced at high level while others are not. Finally we tested whether VHH can be incorporated in microbicides.

In chapter one, we solved the structure of VHH D7, as it would yield valuable information on its mode of interaction with gp120 and would

highlight the role of the different CDRs. D7 did not seem to interact with CD4bs in the same way as other neutralizing human antibodies, like b12. No aromatic amino acids are present on CDR2 and the Y100a on the apex of CDR3 was shown to be in a non-permissive orientation to reach for the Phe43 cavity on gp120, commonly targeted by neutralizing CD4bs-antibodies. On the other hand, the different mode of interaction with gp120 was also suggested by the distinct neutralization phenotypes of D7 and mAb b12. The VHH, contrary to the human monoclonal, did not bind to the clade B mutant gp120 YU-2 109-428 whereas the escape mutation G366E did not seem to affect the interaction with D7.
We also carried out several mutations of amino acids in CDR3, from which the importance of W96 emerged. Taken together, the solution of the structure and the mutational analysis established a firm role of CDR3 and its flexibility in the interaction with, and the neutralization of, HIV-1 and called for the determination of a structural complex including D7 and gp120.

To extend the array of clones targeting the neutralizing epitope hit by A12/D7, we generated synthetic diversity by a PCR-based family-approach described in chapter two. Based on the utmost importance of CDR3 in the interaction with CD4bs, we created a phage library of VHH homologous to the parental A12/D7 and we sought clones with related nucleotide sequences. During selections, elution was done with trypsin rather than specific elution because the whole library was already specific for CD4bs. In this way, phages that might not be eluted by sCD4 were actually eluted.
Forty-nine clones were isolated, with different neutralization phenotypes and a range of varying potencies. Their characterization led us to map the molecular basis of the neutralization phenotypes: it appeared that the last three amino acids of CDR3 in both A12 and D7 dictate whether the antibody will have a broad or narrow neutralization profile. Mutations in the scaffold and at other positions in CDRs were found, that further contributed to the fine tuning of the neutralization potency.

Next to this approach we have undertaken an alternative strategy aimed at characterizing and improving the neutralization properties of another promising molecule, the newly isolated VHH 1B5. We have designed simple

synthetic libraries that semi-randomize the amino acid diversity in single CDRs and after carrying out selections on these, we have looked for sequence patterns in the output clones. Subsequently we have combined the results obtained in larger libraries called combinatorial beneficial mutation (CBM) libraries, where multiple CDRs were mutated at the same time. Selections were carried out by capturing phages in-solution, with picomolar concentrations of biotinylated antigens, and chasing them for up to 50 hours with un-biotinylated antigens. An alternation of glycoproteins from clade A, B and C was used in subsequent rounds. This *in vitro* maturation had the objective of isolating new clones, derived from 1B5 and not present in the *in vivo* maturated pool of antibodies, with improved breadth and/or affinity.
Ideally one antibody covering most of the circulating HIV strains with potent inhibition would be the best candidate for a microbicidal drug, but the possibility of formatting VHH as homo- or hetero-multimers gives the chance of developing stronger chimeric molecules. Cocktails of conventional antibodies have already been tested, and showed positive results[6], but the increased avidity of linking together two or more VHH, thus generating synergistic effects[7], could also prove successful.

In chapter three we dealt with the secretion efficiency of VHH. Production of a large number of clones in our laboratory and plenty of literature[8-10] demonstrate that their expression in *S. cerevisiae* is relatively straight forward and on average higher than full-chain conventional immunoglobulins[11]. Nevertheless we found that a relevant number of VHH has actually poor production yield, therefore we looked at sequences and structures in order to find determinants for high or low secretion.
It appeared from our study that the contribution of the C-terminal domain of VHH, namely the J segment, is crucial, especially in combination with the presence of conserved residues in the V segment. Numerous approaches have previously identified amino acids important for the stability or secretion[12-15], but to our knowledge this was the first time that specific germline genes (and the segments that they encode) are associated with secretion efficiency.
The importance of the production system was also stressed, as we suggested that in the endoplasmic reticulum, the presence of specific chaperones plays an

important role in determining the folding rate of the VHH, and has consequence on their secretion efficiency.

In the fourth chapter of this study we addressed (some of) the challenges that are likely to be encountered in the development of a topical microbicide based on neutralizing VHH. Despite using the well characterized A12 and D7, the features we described are independent of specific clones and can be associated more generally with the wider class of llama antibody fragments.

We investigated the compatibility of VHH with formulations commonly used for topical microbicides, such as aqueous gels and vaginally inserted silicon rings. Specific formulations imply different modes of use and application. A coitally-independent product that can be applied easily by the user would be desirable. Once-a-month formulations would most likely increase adherence and decrease the cost-per-dose. In this study we reached a sustained release for as long as one week, but the stability of the tested VHH and improving the load of silicon rings suggest that a longer release time can be achieved. The hydrophilic character of VHH makes them rather soluble in aqueous environment, as opposed to a substantial number of other microbicide candidates, who are highly hydrophobic thus negatively affecting the range of compatible formulations[16-18].

Thermal- and pH-stability have implications in both the use of the product, and its storage. Extended time at stress conditions did not have a relevant effect on the activity which indicates an interesting suitability of VHH-based microbicides in settings where e.g. cold storage is missing.

Important issues still need to be addressed before human trials can be planned. First of all the *in vivo* efficacy in non-human primates has to be demonstrated. Preliminary tests with SHIV strains have shown promising data about the neutralization titers of several VHH (D. Davies, personal communications), which should open the way to the next step. Immunogenicity of VHH is also to be considered and even though experiments previously performed with anti-rotavirus clones demonstrated low tendency to aggregate and low immunogenicity in humans, specific data should be collected for anti-HIV-1 VHH. Possible emergence of escape

mutants should also be accurately investigated, in the perspective of a prolonged use of the microbicide by unknowingly infected users. It should nevertheless be considered that when more than one VHH is used and different epitopes are targeted, the probability that concerted mutations will appear simultaneously is very low.

Certainly of interest for future improvements is the implementation of a defense strategy based on a double-layered protection. Considering the necessary cooperation of a number of actors to allow viral fusion, neutralizing epitopes can be found on both glycoproteins of the viral spike, but also on the cellular receptors involved in these events. On the other hand, it was recently described that anti-CXCR4 VHH demonstrated strong antiretroviral activity[19]. Maybe targeting the virus and the cell at the same time with a cocktail formulation could provide an effective protection.

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Summary

Heavy-chain antibodies are present in llamas next to conventional immunoglobulins. Their variable domain, also known as VHH is a small, single-chain globular protein with full antigen-binding capacity. Its single-domain nature and some structural differences from conventional immunoglobulins make it a tool with high potential for a number of biotechnological and medical applications.

The HIV pandemic represents since three decades a dramatic health problem throughout the world and especially in resource-limited countries. As an alternative to vaccination, prevention is regarded as the best way to control the pandemic, both from a healthcare and economic point of view. The development of microbicides that help preventing sexually transmitted infections of HIV (the most common way the virus is transmitted) is therefore highly desirable. In this thesis, several aspects of the development of llama heavy-chain antibody fragments as active compound of topical microbicides are investigated.

In chapter one the 3D structure of the HIV neutralizing VHH D7 was determined. The extension, flexibility and amino acid-composition of its CDRs were compared to other known (human) neutralizing antibodies in order to define the way D7 neutralizes the virus by binding gp120. Structural comparison suggested diverse modes of interaction. Mutational analysis identified CDR3 as key area of the interaction with gp120, whose conformational flexibility is likely to accommodate multiple modes of antibody binding inside the CD4 binding site.

Comparison of the related VHH A12 and D7 revealed broad and narrow cross-clade neutralization phenotypes respectively. In chapter two a family-specific phage library was generated and a number of homologous VHH was selected with varying neutralizing profiles. Analysis of their sequences and their recognition of a panel of different HIV-1 subtypes, allowed the understanding of the molecular basis of the different neutralization behaviors. In particular the important role played by the last two C-terminal amino acids of CDR3 was demonstrated.

Secretion efficiency of VHH is a particularly important factor when large amounts are needed at low production cost. In chapter three we analyzed databases of structures and sequences to find sequence-related factors that influence the yield of VHH when produced in the host *S. cerevisiae*. We showed how the combined presence of five key residues and specific J segments is fundamental for proper folding. We also suggested that interactions with ER-resident chaperones are necessary to achieve high secretion efficiency.

In the last chapter, VHH A12 and D7 were used as models to study their availability, activity and stability in a VHH-based topical microbicide. We systematically analyzed likely challenges that VHH will have to face in case of use in sub-tropical settings of resource-limited countries, as HIV preventative drug. VHH proved to be readily available when formulated as tablets or aqueous gel and very stable after extended incubation at physiological temperature and at low pH. We also investigated the tissue-penetration kinetics of VHH in order to assess whether the drug would be present in the sub-mucosal layers of the vagina and once there, carry out its protective action.

Samenwatting

De lama bezit naast conventionele antilichamen ook zware-keten antilichamen. Het variabele domein van deze zware-keten antilichamen, ook wel VHH genoemd, is een klein, enkel-strengs, globulair eiwit met volledige antigen bindende capaciteiten. De enkelvoudige domein compositie van de VHHs en de diverse structurele verschillen met conventionele antilichamen, geven deze eiwitten enorme potentie voor biotechnologische tools en medische applicaties.

Al 30 jaar zorgt de HIV pandemie voor gezondsheidsproblemen over de hele wereld en vooral in onwikkelingslanden. Preventie wordt daar, als alternatief voor vaccinatie, vanuit een medisch en economisch standpunt gezien als de beste methode om de pandemie te controleren. De ontwikkeling van microbiciden die helpen seksueel overdragen HIV infecties tegen te gaan is daarom wenselijk. In deze scriptie wordt gekeken naar de ontwikkeling van lama zware-keten antilichaam fragmenten als actieve substantie in microbicide formules.

In hoofdstuk 1 wordt de 3D structuur van de HIV-neutraliserende VHH D7 beschreven. De conformatie, flexibiliteit en aminozuur sequentie van de CDRs zijn vergeleken met andere reeds beschreven humaan neutraliserende antilichamen om te begrijpen hoe D7 door GP120 te binden het virus neutraliseert. Structuur vergelijkingen gaven aan dat er verschillende manieren van interactie waren tussen antigen en antilichaam. Mutatie studies identificeerde CDR3 als het sleuteldomein in de interactie met GP120, die door zijn conformationele flexibiliteit waarschijnlijk op meerdere manieren een interactie aan kan gaan in de CD4 binding site.

De gerelateerde VHHs A12 en D7 geven brede en smalle neutralisatie patronen respectievelijk over de HIV subtypes. In hoofdstuk 2 wordt een familie-specifieke bacteriofaag bank beschreven waaruit verscheidene homologe VHHs geselecteerd waren die in verschillende mate HIV neutraliseren. Analyse van hun sequenties en kruis-specificiteit studies op verschillende HIV-1 subtypes, gaven inzicht in de moleculaire basis van hun

afwijkende neutraliserende karakters; met name de belangrijke rol van de laatste twee C-terminale aminozuren in CDR3.

Secretie efficientie van VHHs is een belangrijke parameter wanneer bulk hoeveelheden geproduceerd moeten worden tegen lage kosten. In hoofdstuk 3 is een analyse van structuren en sequenties beschreven waarin sequentie-gerelateerde patronen gezocht waren die een invloed hebben op de VHH opbrengst in *S. cerevisiae* producties. Wij hebben laten zien dat de combinatie van vijf sleutel residuen en specifieke J-segmenten essentieel is voor correcte vouwing van het eiwit. Ook zijn waarschijnlijk interacties met ER chaperones nodig om hoge secretie efficienties te krijgen.

In het laatste hoofdstuk zijn VHHs A12 en D7 gebruikt als modellen om de stabiliteit, activiteit en beschikbaarheid van VHH gebaseerde microbicide formules te bestuderen. We hebben systematisch de hordes geanalyseerd die VHHs moeten overbruggen in het geval dat ze zouden gebruikt worden in een sub-tropisch derde wereldland als een HIV preventief middel. VHHs waren aangetoond om zowel in tablet als gel formulatie goed te kunnen vrijkomen en waren ook heel stabiel na langere incubatie periodes in fysiologische temperaturen en lage pHs. We hebben ook gekeken naar de weefsel penetratie kinetiek van de VHHs om na te gaan of de VHHs de sub-mucosale lagen van de vagina konden bereiken en, eenmaal daar, een beschermende activiteit kon uitvoeren.

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Andrea

Curriculum Vitae

The author of this thesis, Andrea Gorlani, was born on January 15, 1982 in Milano, Italy. After spending his childhood in Trigolo and Crema, where he received primary and secondary education, he moved to Milano to attend Università degli Studi. He obtained his Bachelor's Degree in Biotechnology in 2004, with a dissertation on the cloning and production of veterinary vaccines in tobacco plants. Subsequently he continued on this track and finished his Master's Degree studies in Industrial and Environmental Biotechnology in 2007, after spending 10 months as an exchange student at Utrecht University studying the optimization of protein secretion in *S. cerevisiae* under the supervision of Dr. Hendrik Adams and Prof. Theo Verrips. This internship got him interested in the production of recombinant proteins, and he therefore accepted the offer of Prof. Theo Verrips to work as a Research Associate within the EMPRO consortium, focusing on the production and purification of llama antibody fragments. Finally, in 2008, a Europrise scholarship allowed him to start his doctoral studies on the development of llama antibody fragments as HIV-1 topical microbicides in Prof. Theo Verrips' laboratory. Since October 2011 he holds a Scientist position in the startup company QVQ BV, located within Utrecht University.

Publications

Book chapters:

Gorlani A, de Haard H, Verrips T. *Expression of Single Domain Antibodies in Saccharomyces Cerevisiae*, in: Methods in Molecular Biology: volume Single Domain Antibodies, Ed. Springer. In print, 2012.

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