Phosphorylation and Ubiquitination in Growth Hormone Receptor Endocytosis and Signalling

Joyce Putters

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Paranimfen: Johan Slotman
Agnes Van Rossum

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Phosphorylation and ubiquitination in Growth Hormone Receptor endocytosis and signalling

Fosforylering en ubiquitinering in groeihormoonreceptor endocytose en signalering
(met een samenvatting in het Nederlands)

Proefschrift

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Joyce Putters

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Contents

Abbreviations

βTrCP	beta-transducing repeat protein
CIS	cytokine-inducible SH2-domain containing protein
DUB	deubiquitinating enzyme
EPOR	Epo-receptor
ER	endoplasmic reticulum
EV	empty vector
FERM	four-point-one, ezrin, radixin, moesin
GH	Growth hormone
GHR	Growth hormone receptor
GHBP	Growth hormone binding protein
GHRH	growth hormone releasing hormone
GHS	growth hormone secretagogue
HECT	homologous to E6-AP C-terminus
IGF	insulin-like growth factor
IκB	Inhibitor of NFκB
Jak	Janus kinase
NFκB	Nuclear Factor κB
MAPK	mitogen activated protein kinase
MVB	multivesicular body
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PRL	prolactin
PTB	phosphotyrosine binding
PTP	protein tyrosine phosphatase
RING	really interesting new gene
SCF	Skp-Cullin-F-box protein
SH2	Src homology-2
SOCS	suppressors of cytokine signalling
STAT	signal transducers and activators of transcription
TACE	tumor necrosis factor-receptor associated factor
UbE	ubiquitin-dependent endocytosis
UBD	ubiquitin-binding domain
UBL	ubiquitin-like protein
UIM	ubiquitin-interacting motif

CHAPTER I

General Introduction

Joyce Putters

Department of Cell Biology and Institute of Biomembranes
University Medical Center Utrecht

Contents Chapter I

Growth hormone

Growth hormone family and structure

Growth hormone (GH), also known as somatotropin or somatotrope hormone, belongs to a class of evolutionary related peptide hormones, e.g. prolactin (PRL) and placental lactogen (PL) (1). Although this class of peptide hormones does not share a high degree of sequential homology, the structures adopt very similar conformations. Furthermore, the structures of other cytokines such as erythropoietin (Epo), several interleukins and leptin are also analogous (2).

Two main splice variants of GH have been identified, one of which contains 191 and the other 176 amino acids. 85% of circulating GH represents the 191 amino acid protein (3) and the structure of this GH variant has been crystallized more than 20 years ago (4). Generally, GH contains four α-helical domains connected by loops (5). In addition, two disulfide bonds were identified, of which the one connecting C53 to C165, is important for GH secretion, binding and biological activity (6).

GH secretion

The origin of GH in the circulation was found first to be the somatotrophic cells in the anterior pituitary gland (7), but to date several studies found that extra-pituitary tissues also produce GH, such as mammary (8), neuronal (9), retinal (10), lung (11), bone (12) and several types of immune cells (13,14). GH-levels in the circulation are regulated by several factors. Two of them are somatostatin, which inhibits GH-release, and GH-releasing hormone (GHRH), which stimulates GH-release. Furthermore, Growth Hormone Secretagogue Receptor (GHSR) induces GH release upon activation by the GH secretagogues (GHS), of which an example is ghrelin (15-17). Another important factor is pituitary transcription factor Pit-1, which regulates all GH synthesis and release, but is also involved in the differentiation and maintenance of GH-producing cells in the pituitary gland (18). Moreover, insulin-like growth factors (IGFs), which are induced by GH, inhibit GH-release, resulting in a negative feedback loop (19).

The outcome of the many factors involved in the regulation of GH-release is a pulsatile GH-release pattern in the circulation (7). It is important to note that the pulsatile secretion pattern of GH is regulated by hypothalamic activity and is primarily aimed at the pituitary. The secretion regulation of other GH-producing cells, like in mammary tissue, is less well understood and not pulsatile. Therefore, female GH-secretion is not as strongly pulsatile compared to male GH-secretion (20,21), which results in sexual dimorphisms in several processes, including hepatic metabolism (22,23). GH has a half-life of about fifteen to twenty minutes and clearance occurs in the kidneys and by internalisation via the GH receptor (GHR). GH binding protein (GHBP), representing the extracellular domain of the GHR (24), circulating in the bloodstream is able to bind GH and prevents GH from being cleared from the circulation (25-27). In this way, GHBP can increase GH levels in the bloodstream, although this mechanism could also serve to let cells only respond to high GH levels that exceed the level of GHBP. Normally, about 50% of total GH in the bloodstream is in complex with GHBP (28).

Clinical relevance of GH

GH actions involve multiple organs and systems and are essential for postnatal longitudinal growth and development. Originally, it was postulated that all effects of GH were mediated by IGF1, which is known as 'the somatomedin hypothesis'. His turned out not to be the case. Moreover, IGF1 expression is stimulated by many other factors than GH. IGF1's most important role is in mediating the anabolic and linear growth promoting effect of GH (29). In addition to promoting postnatal longitudinal growth, GH is responsible for changes in protein, lipid and carbohydrate metabolism. Shortly, under starving conditions, GH changes consumption from carbohydrates and protein to lipids, which allows conservation of vital protein stores (30).

GH hypersecretion results in acromegaly or gigantism, a condition associated with significant morbidity and mortality. Clinical features include coarse facial features, large, spade shaped hands and large feet. In contrast, GH hyposecretion results in dwarfism with clinical symptoms as reduced lean body mass, increased fat mass, impaired systolic function and impaired quality of life (29).

Apart from GH secretion, defects in GHR, post-GHR receptor signalling or primary defects in IGF1 synthesis result in phenotypes resembling GH hyposecretion, varying from more or less severe. The first well-described clinical phenotype of severe growth failure was described by Laron and coworkers and therefore named the Laron syndrome (31). They initially searched for a defect in the GH protein and its secretion, but they found eventually that GH could not bind to the GHR due to deletions or mutations in the receptor (32,33).

Growth hormone receptor

Cytokine superfamily

The GHR belongs to the class I cytokine superfamily, including PrL receptor (PrLR), erythropoetin receptor (EpoR), thrombopoetin receptor (TpoR), ciliary neurotrophic receptor, leukaemia inhibitory factor, several interleukin receptors, oncostatin M receptor, granulocyte colony stimulating factor and leptin receptors (34). Receptors for interferon $\alpha/\beta/\gamma$ and IL-10 are classified as class II cytokine receptors and are more distantly related (35).

The cytokine superfamily shares several important structural features, which can be found in the extracellular as well as in the intracellular part of the proteins. Importantly, receptors of the class I cytokine receptor superfamily lack intrinsic kinase activity (36). In the intracellular domains of class I cytokine receptors two motifs with limited amino acid homology were identified: box-1 and box-2 (36). Box-1, a proline-rich region, consisting of eight amino acids, is present in all members and is important for binding of the family of Janus kinases (Jak2) that provide kinase activity to the cytokine receptors (37,38). This motif consists of a P-X-P (P, proline; X, any amino acid) sequence and is preceded by a region of hydrophobic amino acids (39,40). Box-2 is only 50% conserved in the class I cytokine receptor family and is present in most, but not all receptors. The latter motif contains a stretch of hydrophobic amino acids followed by negatively charged residues and one or two basic amino acids (41). Their extracellular domains include two fibronectin type III-like extracellular domains, four conserved cysteines and a tryptophan-serine-X (any amino acid)-tryptophan-serine (WSXWS) motif (42,43).

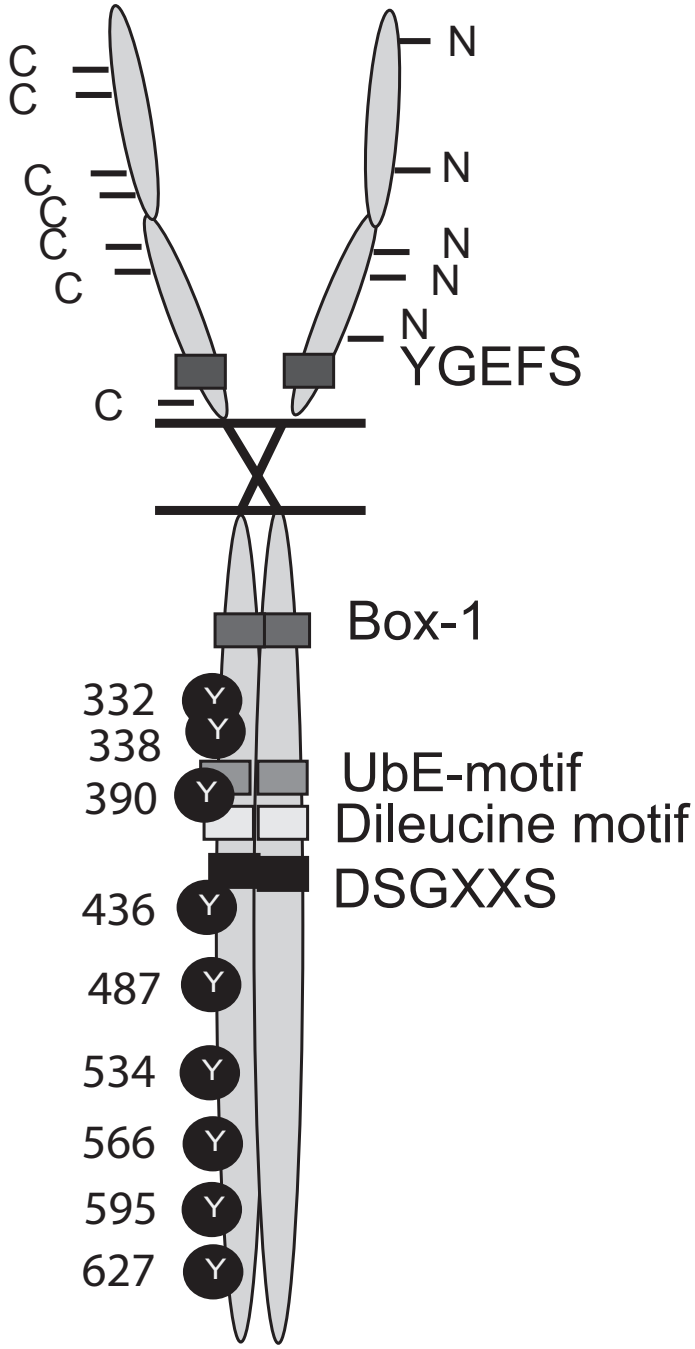

Figure 1: Rabbit GHR structure
The rabbit GHR contains several important residues and motifs, including five potential glycosylation sites (N), seven cysteine residues (C), the YGEFS motif, the box-1 region, the UbE-motif, the DSGXXS motif and nine tyrosines (Y).

GHR structure

Cloning of rabbit and human GHRs in 1987 revealed a 84% sequence homology between the two species (24). Subsequent cloning of the GHR cDNA from rat, mouse, cow, sheep, pig and chicken showed that the overall sequence conservation was over 50% (44). The GHR is synthesised with an 18-amino acid signal peptide. The mature GHR consists of 620 residues, of which the extracellular part contains 246, the transmembrane part 24 and the intracellular part 350 amino acids (Fig. 1) (24).

The extracellular domain of the GHR in complex with GH has been crystallized in 1992, which showed that only one GH molecule binds to a GHR dimer (Fig. 2). In the complex, both receptors interact with essentially the same residues of GH, although the two binding sites on GH have no structural similarity (45).

The GHR extracellular domain contains seven cysteine residues, of which six form disulfide bridges (46). Moreover, five potential N-linked glycosylation sites are present, which are not involved in GH binding (24). N-linked glycosylation is responsible for the difference in apparent molecular mass between the precursor GHR in the ER (M_r= 110.000) and the mature glycosylated GHR at the cell surface (M_r= 130.000). Furthermore, the conserved WSXWS-motif is present in the GHR as YGEFS. This motif does not physically interact with GH, but is structurally important for GH binding affinity and GHR signal transduction (47).

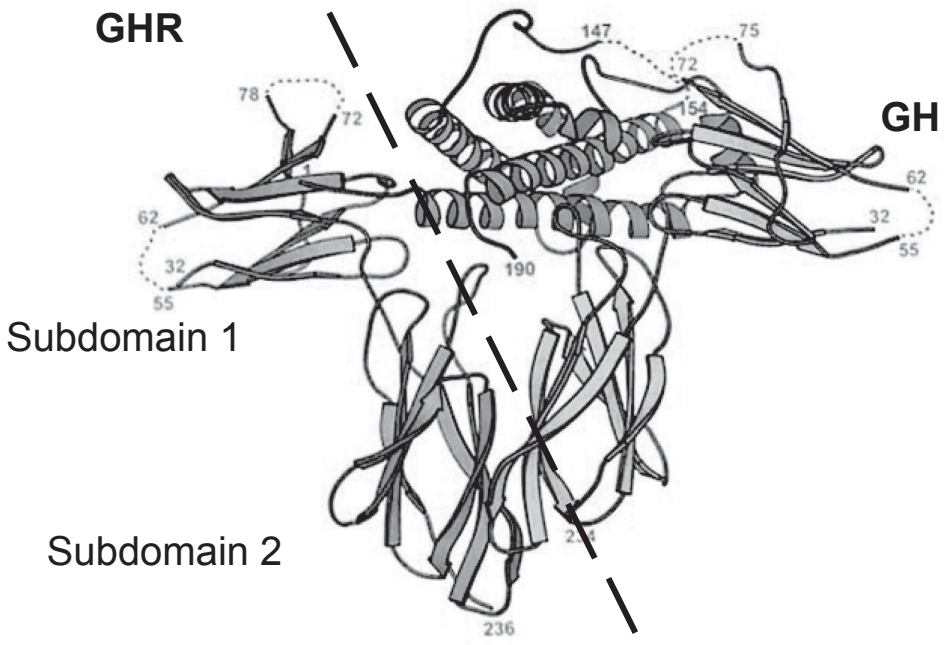

Figure 2: Structure of the GH-(GHR)2 complex
Crystal structure of part of the GHR dimer in complex with one molecule of GH, as crystallised by de Vos et al (45). The dashed line separates GH from GHR.

The rabbit GHR intracellular domain contains nine tyrosines, which can be potentially phosphorylated upon GHR activation. In addition, several functional motifs are present in the cytosolic tail. The membrane-proximal box-1 motif in GHR is located between amino acids 293 and 304 (48). Within box-2, a motif important for GHR endocytosis was discovered: the Ubiquitin-dependent endocytosis (UbE) motif (49). The GHR also harbours a classical DSGXXS degradation motif, although the functionality of this motif has not been shown yet. Finally, a classical dileucine motif (DTDDRLL) is present that functions as an endocytosis motif via adapter protein complex 2 (AP2) and clathrin, only if the GHR is truncated at residue 367 (50).

GHR life cycle

After translation of the GHR mRNA on ribosomes, the native GHR is translocated into the endoplasmic reticulum (ER) by means of its signal peptide (24). In the oxidative environment of the ER, the disulfide bonds form and the GHR already forms dimers. The finding that the GHR arrives at the cell surface as a preformed dimer was found originally by Gent et al (51). Comparison of the crystal structures of the liganded GHR (45) and the unliganded GHR confirmed that a conformational change induced by GH binding initiates signal transduction, rather than dimerisation (52). In the ER, the receptor is N-glycosylated via dolichol. After correct folding, the GHR is allowed to leave the ER and is transported to the Golgi apparatus, where all high-mannose oligosaccharide groups are modified into complex oligosaccharides. Trafficking of the GHR continues towards the cell surface (Fig. 3).

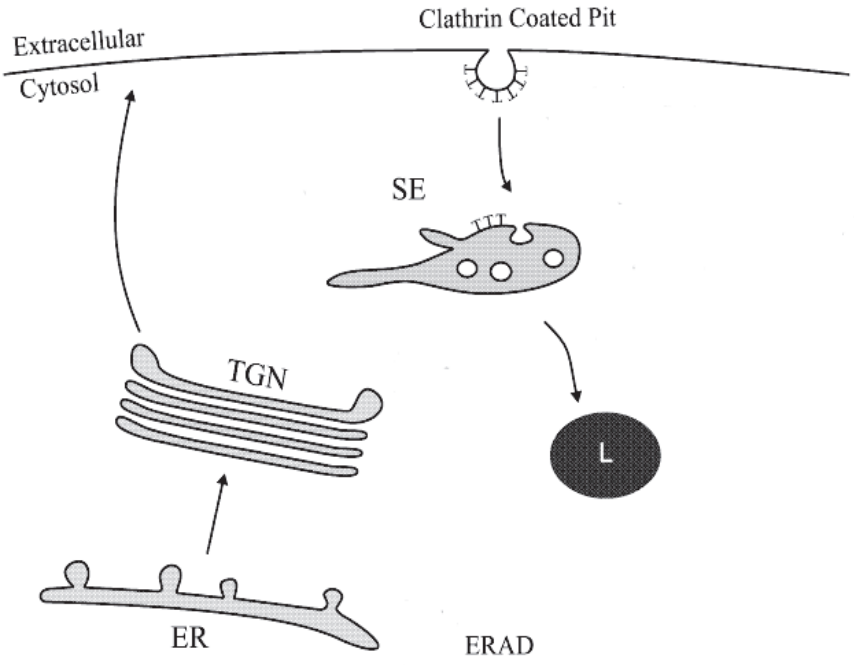

Figure 3: Life cycle of the GHR

The route from synthesis to degradation of the GHR. The GHR is synthesised in the ER, where the GHR is N-glycosylated. In the Golgi apparatus, the high-mannose oligosaccharide groups are modified into complex oligosaccharides. Subsequently, the GHR arrives at the cell surface as a preformed dimer, where it can bind GH and initiate signal transduction. At the same time, the GHR is downregulated constitutively independent of GH.

At the cell surface, the GHR is endocytosed constitutively independent of its ligand GH (53-55). Before endocytosis, two events might occur to the GHR. Firstly, GH can bind its receptor and signal transduction will be initiated. Secondly, the extracellular domain of the GHR can be proteolytically cleaved by tumor necrosis factor-α-converting enzyme (TACE). In this process, also known as shedding, the extracellular domain is released into the bloodstream (referred to as GHBP) and the intracellular domain is endocytosed and degraded (56). The GHR is internalised by clathrin-mediated endocytosis (57,58), after which the receptor is transported to the endosomal system, sorted into multivesicular bodies (MVB) and degraded in the lysosome (59).

The ubiquitin proteasome system

Ubiquitin

Protein degradation is a tightly-controlled and well-regulated process, allowing rapid removal of regulatory proteins with consequent and immediate effects on important biological processes within the cell. Initially, the endosomal-lysosomal system was discovered as the primary protein degradation machinery, but in the mid-1970's another cellular protein degradation system was identified: The ubiquitin-proteasome system. In this system, ubiquitin is attached to a specific substrate that results in targeting to and degradation by the proteasome (60). The ubiquitin proteasome system was discovered by Aaron Ciechanover, Avram Hershko and Irwin Rose for which they received the Nobel Prize for Chemistry in 2004. The discovery explained the requirement for energy to degrade proteins, which was shown in 1953 by Melvin Simpson (61).

Ubiquitin is a small protein of 76 amino acids and its importance is reflected by the fact that only three amino acids are different between mammals, plants and yeast (62). The structure of ubiquitin is known as the 'ubiquitin fold' or 'ubiquitin superfold' and is characterized by a compact globular fold. Essential for the linkage of ubiquitin to target proteins is the presence of an exposed C-terminal tail (63).

The function of protein ubiquitination

Ubiquitination was initially discovered as an essential mediator of protein degradation by the proteasome, but in the mean time it is clear that ubiquitination is important for a broad variety of cellular processes (64).

The classical view is that monoubiquitination is involved in protein trafficking, endocytosis and gene expression, whereas polyubiquitination is predominantly involved in proteasomal degradation (65). At present, we know that the fate of a polyubiquitinated protein is not always proteasomal degradation. Moreover, adding ubiquitin chains to a substrate may result in DNA damage tolerance, kinase activation, protein trafficking and translation besides proteasomal degradation (62).

One important determinant of what happens after polyubiquitination of a substrate is the type of ubiquitin linkage that is used. Ubiquitin contains seven lysines (K6, K11, K27, K29, K33, K48 and K63), which can all be used to link an additional ubiquitin to the already conjugated ubiquitin. Furthermore, one ubiquitin chain can have different linkage types (62).

The mechanism of protein ubiquitination

The attachment of ubiquitin to a particular substrate generally requires three steps (Fig. 4). Step 1 involves the ATP-dependent activation of the C-terminal glycine residue of ubiquitin by the ubiquitin activating enzyme (E1) and subsequent binding of ubiquitin to a cysteine residue of E1 in a thiolester linkage. The second step involves the transfer of ubiquitin from E1 to an active site cysteine residue of a ubiquitin conjugating enzyme (E2). In the final step, ubiquitin is linked by its C-terminus in an amide isopeptide linkage to an ε-amino group of the substrate's lysine residue by a ubiquitin ligase (E3).

Since the E3 recognizes and binds the substrates, the E3 is considered to be the specificity factor of the ubiquitin system (66). At present, on the basis of structural similarities, two main classes of E3's are known: E3 ligases containing a homologous to E6-associated protein C-terminus (HECT) domain, a really interesting new gene (RING)-finger domain or a U-box (67).

Two different types of mechanisms are known to transfer ubiquitin to the substrate: A direct and an indirect transfer. Direct transfer means that ubiquitin is directly transferred from the E2 to the substrate, whereas the indirect transfer requires the ubiquitin to be transferred first to the E3 and subsequently to the substrate. The direct transfer is usually performed by RING and U-box E3s, whereas HECT E3s mostly transfer ubiquitin indirectly (62). A recent study describes that several ubiquitin binding domains (UBD)-containing proteins can be monoubiquitinated in vitro independent of E3 ligase activity. In that case, the ubiquitin-charged E2 are directly recruited by the UBD of a protein (68). After the linkage of the first ubiquitin to the substrate, a chain of ubiquitin molecules might be formed by subsequent linkage of new ubiquitin molecules to one of the seven ε-lysine residues of the previous ubiquitin (60). A factor which facilitates ubiquitin chain assembly is called E4. E4 binds to monoubiquitinated substrates and is able to conjugate

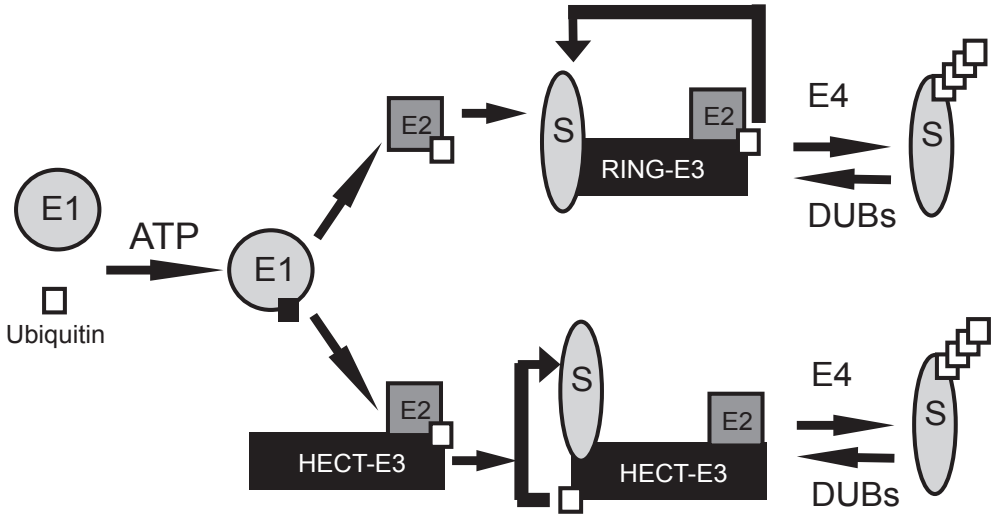

Figure 4: General mechanism of protein ubiquitination.
Schematic representation of protein ubiquitination by RING-type E3s and HECT-type E3s. See text for detailed explanation. S= Substrate

additional ubiquitin molecules to the ubiquitin on the substrates (69).
At present, two different E1 enzymes have been found, namely UBA1 and UBA6. In contrast, dozens of different E2 and hundreds of E3 enzymes have been already identified, which allows specific ubiquitination of target proteins (70).

SCF complexes

History

The identification of the Skp1-Cullin-F-box (SCF) complex as a ubiquitin ligase was initiated with the analysis of cell division cycle (*cdc*) mutants of the budding yeast *Saccharomyces Cerevisiae* (71). It was found by genetic and biochemical studies that Cdc53, Cdc4, Skp1 and Rbx1 associate into a ubiquitin ligase complex and regulates the G1/S transition together with the ubiquitin conjugating enzyme Cdc34 (71-74). Skp1 was already known to interact with Skp2, Cdc4, yeast Ctf13 and human cyclin F (75-77). Sequence alignment of these Skp1 interacting proteins revealed a motif of about forty residues with sequence similarity that is currently known as the F-box (76).
The degradation of proteins by such a complex was dependent on both common and divergent components. This observation led to the F-box hypothesis, stating that Skp1 binds F-box proteins into a ubiquitin ligase complex and that the F-box proteins recruit the substrates (76,78,79). To date, sixty-nine different F-box proteins have been identified in humans and the recognition of phosphorylated substrates confirmed that this complex is important in the cell (67).

Structure and regulation of the SCF complex

Many studies have been undertaken to determine the structure of the SCF complex in order to find evolutionary relationships, the enzymatic mechanism and the basis for substrate recognition (80). The subunits of a typical SCF complex are cullin, Skp1, Rbx1 (a RING finger protein), a F-box protein and an E2 enzyme (Fig. 5) (81).
Cullins are evolutionary conserved from yeast to mammals and seven cullins (Cullin 1, 2, 3, 4A, 4B, 5 an7) and two proteins with a cullin homology domain have been identified (82-86). Together with Rbx1 and the E2 enzyme, cullins form the catalytic core, while Skp1 connects the N-terminus of the cullin to the F-box protein via interaction with the F-box (87). The N-terminus of the crystal structure of cullin revealed a helical domain that is composed of three repeats of five-helix bundles. Skp1 binds to the first N-terminal repeat (88,89). The C-terminus of cullin is composed of a four-helix bundle, an α/β domain and winged-helix B (WHB) subdomains which recruit Rbx1 (88,90).

Mechanism and regulation of SCF complex activity

The crystal structure of SCF^{Skp2} revealed a large gap of about 50 Å between the E2 catalytic site and the substrate (81,91,92). For a long time, it was unclear how a rigid complex could overcome a gap of 50 Å, until it was found that neddylation of cullin results in a conformational change of the C-terminus of cullin (93-95). This conformational change abrogates the interaction of Rbx1 with the WHB subdomains of cullin, resulting in a flexibly cullin-linked Rbx1, which is able to adopt various conformations (96). Neddylation is the covalent attachment of NEDD8 to a protein. In the case of cullin, NEDD8 is attached to a conserved lysine in the C-terminus of cullin. It has been shown that cullin neddylation

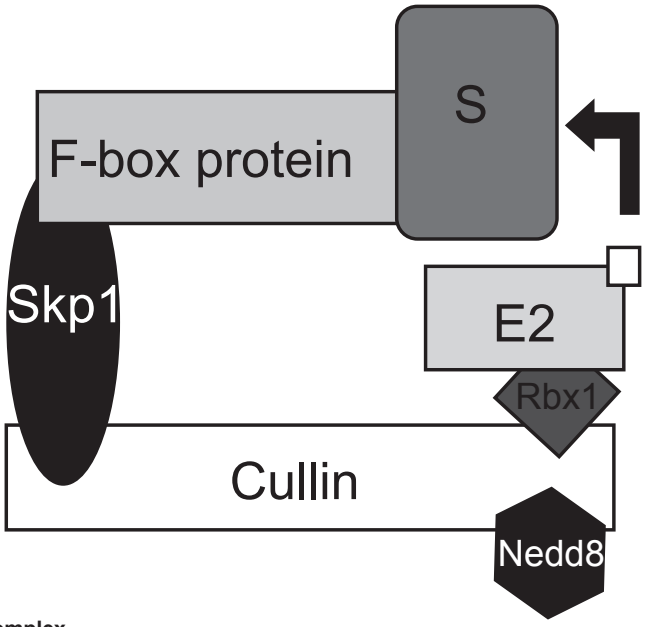

Figure 5: The SCF complex
Schematic representation of the general components of a SCF complex. See text for detailed explanation. S= substrate, U= Ubiquitin.

increases the affinity of $SCF^{\beta TrCP}$ for the ubiquitin-loaded E2 UBC4. Moreover, NEDD8 interacts with UBC4 (97,98). On the other hand, NEDD8 has been found to stimulate ubiquitin transfer from E2 to the substrate lysine. Both transfer of the first ubiquitin and ubiquitin chain elongation are stimulated by neddylation (99,100). In addition to neddylation of cullin, dimerisation of the SCF complex via the dimerization (D) domain in the F-box protein is essential for SCF activity (101). Recently, new insights into protein ubiquitination by SCF complexes and E2 enzymes were presented. The interaction between SCF and E2 Cdc34 is very dynamic, illustrated by a rapid E2-E3 disassembly and assembly. This property is a consequence of the acidic tail in the E2 and a basic 'canyon' in cullin and facilitates ubiquitin chain assembly. The acidic tail in the E2 and the basic 'canyon'in cullin were shown for Cdc34 and Cul1, but considering that the region in Cul1 in conserved in Cul1 orthologs and paralogs, it is suggested that this type of dynamic interaction between E2 and E3 stimulates ubiquitin chain assembly in all cullin-RING ubiquitin ligases (102).

The finding that cullin neddylation and SCF complex dimerization are essential for SCF complex activity means that these two processes must be tightly controlled. The Cop9 signalosome has been found to hydrolyse NEDD8, thereby inactivating the SCF complex. In addition, a protein called CAND1 sequesters non-neddylated cullin1-Rbx1 complexes and prevents thereby SCF complex assembly. Several reports also state that the availability of the adapter (Skp1) bound to a F-box protein triggers SCF complex assembly and its activation through neddylation of cullin *in vitro* (103,104). Specific cues have also been shown to regulate SCF activity. For example, UV irradiation rapidly activates SCF^{DDB2} (105). Finally, a recent report described that phosphorylation events during the cell cycle were able to regulate SCF complex activity (106).

$SCF^{\beta TrCP}$

One of the best characterised F-box proteins is β-transducing repeat-containing protein (βTrCP), which was first identified as an interaction partner of HIV-1 Vpu (107). βTrCP is member of the F-box/WD40 repeat containing (Fbw) proteins, which have a 42-48 amino-acid F-box motif at the N-terminus and seven WD40 repeats, structured as a seven-bladed propeller at the C-terminus (108). For HIV-1 Vpu, it has been shown that although the first WD40 repeat of βTrCP is most important for binding to βTrCP, all seven WD40 repeats are required for optimal binding (109).

The consensus sequence for substrate binding by βTrCP is the $DSG(X)_{2+n}S$ motif, where the serines must be phosphorylated. Optimally, a lysine located at 9-13 residues upstream of the motif should be present (108).

$SCF^{\beta TrCP}$ has been shown to be involved in major regulatory mechanisms such as cell cycle progression, metabolism, development and immunity (110). Important and well-characterised substrates are IκB, the inhibitor of NFκB, and β-catenin, a key factor in Wnt signalling, which are both recognised by βTrCP via the phosphorylated consensus destruction motif (67). Under normal conditions, NFκB is present in the cell bound to IκB, preventing NFκB from translocation to the nucleus. Upon phosphorylation of IκB, IκB is recognised and ubiquitinated by $SCF^{\beta TrCP}$ followed by degradation by the proteasome, which allows NFκB translocation to the nucleus and stimulation of gene transcription (111). Many different cancers have been associated with enhanced constitutive activity of NFκB (111,112), which might be the result of increased $SCF^{\beta TrCP}$ activity (108). In non-stimulated cells, β-catenin is phosphorylated, recognised by $SCF^{\beta TrCP}$, ubiquitinated and degraded. Upon Wnt stimulus, β-catenin is stablised and translocates to the nucleus to stimulate transcription of its target genes (113-117). The β-catenin pathway is associated with many malignancies, such as colorectal cancer, hepatocellular carcinomas and malignant melanomas, in which often β-catenin is constitutively not degraded (108).

Deubiquitination

Ubiquitination is a reversible process, indicating that, like phosphorylation, it is a powerfool regulatory tool in cell signalling. The cysteine proteases responsible for the process of deubiquitination are called deubiquitinating enzymes (DUBs). DUBs deubiquitinate by catalysing the hydrolysis of the isopeptide bond between ubiquitin and itself or a target protein.

Like phosphatases in phosphorylation-based regulation of proteins, DUBs are important for the proper functioning of the ubiquitin system in several ways (118). As ubiquitin expression occurs always as a fusion of ubiquitin with one or two ribosomal proteins or as a linear polyubiquitin, they need to be cleaved to generate single ubiquitin moieties, which requires DUBs. In addition, DUBs also remove an extra residue at the C-terminus from the ubiquitin proprotein to generate a free C-terminal glycine residue (119-122). Recycling of ubiquitin is another important function of DUBs. Ubiquitin may be trapped by the reaction of cellular nucleophiles with the thiolester intermediates involved in protein ubiquitination. By deubiquitination, these ubiquitin molecules are allowed to be attached to a protein substrate (123). A third role of DUBs is to antagonize E3 ligase activity by deconjugating selective substrates. This role is similar to the antagonising role of phosphatases towards kinases and this phenomenon allows rapid regulation of the functional state of proteins (118,124). Finally, DUBs cleave unanchored polyubiquitin into

functional mono-ubiquitin. These poly-ubiquitin chains are either synthesized *de novo* by the conjugating machinery or have been released from substrates by DUBs (125,126).
The first human disease associated with a defect in a DUB was cylindromatosis (127). Familial cylindromatosis is a rare, autosomal dominantly inherited predisposition to tumors of the skin appendages. It has been shown that a defect in the deubiquitination activity of the cylindromatosis gene (CYLD) is the result of this disorder (128-130).

Ubiquitin-like proteins

Analogous to ubiquitin, eukaryotic cells contain more small proteins, containing a ubiquitin-fold, that can be conjugated to protein substrates via their C-terminal amino acid. Thereby, they influence cellular processes such as cell division, immune responses and embryonic development. These proteins are referred to as ubiquitin-like modifiers (UBLs) and also use E1-E2-E3 systems. As a consequence of the diversity of their functions, defects in UBLs can result in diseases like cancer neurodegenerative disorders and muscle atrophy or cachexia. To date 17 UBLs are identified, including SUMO, NEDD8, ISG15 and FAT10 (70). SUMO has been described to function in protein localisation and activity, but can also serve to mark proteins for ubiquitination (131). NEDD8 is important for the activation of cullin-based E3s, as discussed above (90,95,132), whereas ISG15 and FAT10 conjugation are controlled by the interferon system, which responds to viral signals (70).

The ubiquitin system and disease

The consequence of the involvement of the ubiquitin system in many important cellular pathways is that malfunction of this system frequently results in disease like cancer, viral infection, neurodegenerative disease, metabolic disease and muscle wasting (133).
Mutations in ubiquitination factors involved in the regulation of cellular proliferation have been described to cause several types of cancer. Mutations in BRCA1, which forms together with BARD1 an E3 ligase complex, are associated with the inherited predisposition for breast and ovarian cancer (134-136). Some of the cancer-predisposing mutations in BRCA1 result in loss of E3 ligase activity *in vitro* (137-139).
Viruses hijack the ubiquitin system to modify the cell to promote virus replication and immune response evasion. One example is the Human Immunodeficiency Virus (HIV), which encodes two proteins (VIF and VPU) that interact with distinct cullin-RING based host E3s to promote ubiquitination and degradation of cellular proteins (133).
Neurodegenerative disease has been associated for a long time with the formation of protein aggregates. In Huntington's disease, polyglutamine repeat expansion in proteins promotes the formation of protein aggregates that cannot be degraded in the proteasome and impairs its proper function (140).
Diabetes is an example of a metabolic disease associated with the ubiquitin system. Although not yet completely understood, it has been suggested that suppressor of cytokine signalling proteins (SOCS)-induced insulin receptor substrate (IRS) 2 degradation, presumably via the elongin BC ubiquitin-ligase, contributes to a general mechanism of inflammation-induced insulin resistance (141).
During muscle wasting conditions, a high number of genes related to the ubiquitin system are upregulated, including a muscle-specific ubiquitin ligase and a potential substrate receptor SCF (142). Deletion of these proteins results in decreased muscle mass, but the exact mechanism is not yet known.

GHR signalling

Turning the stimulus into a signal

Signalling of the GHR starts with binding of one GH molecule to a preformed GHR dimer, resulting in a conformational change of the GHR (52). Consequently, the two Jak2 molecules bound to the two box 1 motifs of the GHR dimer come into close proximity and contact each other (143). Subsequently, the Jak2 molecules phosphorylate each other, followed by phosphorylation of the tyrosine residues of the GHR cytosolic tail. Jak2 activation lasts for about two to three minutes, whereas GHR phosphorylation can be detected for at least twenty minutes (144). So far, Jak2 is the only tyrosine kinase that is known to phosphorylate the GHR. However, a recent *in vivo* study showed that the GHR does not exclusively signal through Jak2. GH, injected in mice, expressing a GHR with a disabled box-1, can activate Src and ERK1/2 but not Jak2, STAT3, STAT5 and Akt (145).

Jak family

The Jak family of non-receptor tyrosine kinases consists of four members: Jak1, Jak2, Jak3 and Tyrosine kinase 2 (Tyk2) (146). Jak1, Jak2 and Tyk are widely expressed in mammalian cells, whereas Jak3 occurs mainly in hematopoetic cells (147,148). Jak family members localise to the plasma membrane and endosomes together with their cognate receptors. Upon overexpression in experimental conditions, Jak can be found in the cytosol (146,149). Jak1 knockout mice have a perinatal lethal phenotype, probably because the mice have neurological defects that prevent them from suckling (150). Jak2-/- mouse are embryonically lethal, because of failure in erythropoiesis (151,152). In contrast, Jak3 deficiency is not lethal, but results in severe combined immunodeficiency (SCID) (153,154).

The crystal structure of none of the members of the Jak2 family has been solved yet, probably because the Jaks are large proteins of more than 1,100 residues. Seven Jak homology domains are identified that are conserved among mammalian, avian and insects, referred to as JH1 to JH7, counting from the C-terminus (Fig. 6) (146). The JH1 domain is the kinase domain, whereas the JH2 domain comprises the pseudokinase domain. The domains JH3 and -4 make up a SH2-like domain and the JH6 and -7 domains constitute the FERM (Band-4.1, ezrin, radixin, moesin) homology domain (146).

Several tyrosine residues within Jak have been identified that are regulating Jak kinase function. Phosphorylation of tyrosine 119 in the FERM domain abrogates the interaction between Jak2 and cytokine receptors (155) and phosphorylation of tyrosine 317 has been shown to have a role in the inhibition of Jak2 activity (156). In addition, within the pseudokinase domain phosphorylation of tyrosine 570 and serine 523 have been shown to inhibit Jak2 kinase activity (157,158) and phosphorylation of tyrosine 637 is important for enhancing Jak2 activity (156). Furthermore, within the kinase domain phosphorylated

Figure 6: Jak2 structure
Jak2 consists of a FERM, SH-2, pseudokinase and kinase domain, which are schematically shown in the figure.

tyrosine 813 enhances Jak2 signalling by recruitment of SH2-B/SH2B1 (159), whereas phosphorylated tyrosines 1007 and 1008 are essential for kinase activation (160). Finally, phosphorylation of tyrosine 913 inhibits Jak2 signalling (161).
Three regions within the pseudokinase domain have been shown to regulate basal Jak2 activity through an autoinhibitory mechanism. The inhibitory function is accomplished by a relatively weak interaction of the pseudokinase domain with the kinase domain. Upon cytokine stimulation, the interaction between pseudokinase and kinase domain is abrogated and is possibly replaced by the interaction of the kinase domains of the two Jak2 molecules present on the receptor dimer (162,163). Recently, it was found that the linker connecting the SH2 domain and the pseudokinase domain is essential for Jak2 catalytic activity (164).
The FERM domain of the Jaks is essential for binding to cytokine receptors (165,166). In addition, the FERM domain has been found to interact with the kinase domain of Jak2 and enhancing Jak2 kinase activity (167,168).

GHR signal transduction pathways

GH stimulation of the GHR results in the activation of several signal transduction pathways, including the signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, the Insulin Receptor Substrates (IRS), the phosphoinositide-3 kinase (PI3K) pathway, the NFκB pathway and the protein kinase C (PKC) pathway (Fig. 7) (169).

The STAT pathway

The human family of STAT proteins consists of seven members and are all activated by multiple growth factors and cytokines (170). GH has been shown to activate STATs 1, 3, 5A and 5B via Jak2 kinase activity. STAT1 and 3 both bind the c-sis-inducible element (SIE) and activate the *c-fos* gene. The STAT5A and B isoforms activate transcription of IGF-1 and serum protease inhibitor 2.1 (spi2.1) by binding the GH responsive element (GLE). Serine-threonine kinase Akt-1 interacts with both STAT5A and B (7).
Different receptors recruit different STAT proteins, which largely depends on the binding affinity of the SH2 domain of the STAT for a specific phosphorylated tyrosine in the GHR (171). Once a STAT protein is bound to the GHR, Jak2 is able to phosphorylate the STATs, resulting in dissociation from the GHR. Subsequently, the STAT proteins form dimers and translocate to the nucleus where they stimulate their target genes.
STAT knockout mice have been used to identify the STATs involved in GHR signalling. The body growth of STAT1 knock out mice was not affected (172), whereas the STAT3 knockout mice were embryonically lethal, meaning that STAT1 is not a major determinant of body growth and that the role of STAT3 was impossible to determine (173,174). STAT5A-deficient mice were affected in mammary gland development and lactogenesis, which are both processes regulated by the prolactin receptor. Body growth, serum IGF-1 levels and expression of GH-regulated genes were only modestly affected in both male and female STAT5A knockout mice (175,176). Importantly, STAT5B deficient mice were found to have strong body growth phenotypes, especially male knockout mice were significantly smaller. Interestingly, STAT5B-deficient males grew similarly as female wild type and knockout mice, demonstrating that STAT5B is most important for transducing the sexual dimorphic GH secretion pattern into a sex-specific pattern of body growth (176,177).

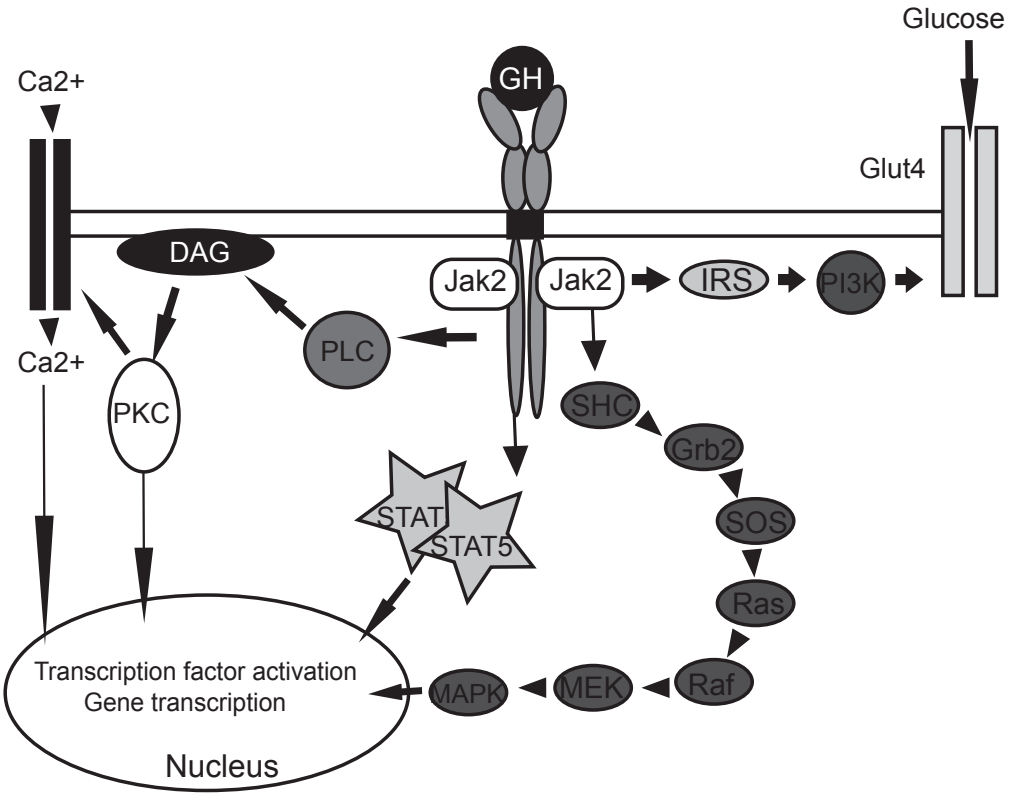

Figure 7: GHR signal transduction routes
Upon GH stimulation, the GHR is phosphorylated by Jak2, resulting in activation of STAT, MAPK, PI3K and protein kinase C signal transduction pathways, which lead to stimulation of transcription of target genes.

For STAT5B, some GHR cytosolic tyrosines are required for its activation. Tyrosines 534, 566 and 627 in porcine GHR (same residues in rabbit and human GHR) were found to be required for GH-dependent STAT5B phosphorylation (178). Furthermore, tyrosines 333 and/or 338 (332 and 337 in rabbit and human GHR) were also suggested to play a role (179).

The MAPK pathway

Another pathway that is activated by a phosphorylated GHR is the MAPK pathway. MAP kinases are serine/threonine/tyrosine kinases that regulate cellular growth and differentiation (180). In mammalian cells, three important MAPK pathways have been identified: ERK (also referred to as classical MAPK), C-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38 kinase. Each MAPK cascade involves the sequential action of at least three enzymes, which allows strong signal amplification (181). The ERK pathway is best-characterized and is activated by growth factors and cytokines, including GH. GH stimulation results via Jak2 and GHR phosphorylation in the phosphorylation of SH2 domain-containing transforming protein C (Shc). Phosphorylated Src recruits the adapter protein growth factor receptor-bound protein (Grb2). In addition, the cascade involves a guanine nucleotide exchange protein called Son of Sevenless (SOS), a small GTP binding protein $p21^{ras}$. Subsequently, Ras activates

a cascade including sequential activation of Raf and MEK, which ultimately results in the activation of ERK1 and 2. Phosphorylated ERK translocates to the nucleus, activates several transcription factors and induces cellular responses, of which transcriptional activation of the immediate early gene c-fos is most important (181,182).
The JNK pathway is involved in many cellular processes, such as cell proliferation, cell differentiation, apoptosis and survival signalling. Activators of JNK include stress, cytokines, growth factors and ceramides. A similar activation cascade as described for the ERK proteins is needed for JNK activation, which has substrates like c-Jun, activating transcription factor 2, Elk-1, p53 and Sap-1a.
Mammalian p38 MAPK is activated by cellular stresses like UV radiation, heat shock, osmotic stress, proinflammatory cytokines and certain mitogens. An important substrate of p38 MAPK is phospholipase A2, which is required for interferon signalling, but p38 MAPK is involved in other cellular processes such as NFκB, STAT1 and many more. It has been shown that GH activates all three MAPK pathways described above (183,184).

IRS, PI3K and NFκB
IRS1, 2 and 3 are phosphorylated by GH and proposed to mediate the insulin-like effects of GH (185). PI3K is either a direct target of GHR or it binds to the phosphorylated tyrosines of IRS and is activated, but not phosphorylated upon GH stimulation (186). PI3K has been associated with DNA synthesis, glucose uptake, activation and inhibition of apoptosis via Akt serine threonine kinase (187,188). PI3K activation contributes to the insulin-like effects of GH by inducing translocation of the glucose transporter 4 to the cell surface, which results in the stimulation of glucose uptake (7).
Another important cellular pathway that is affected by GH stimulation is the NFκB pathway (189) that can be regulated via IP3K and its downstream target Akt (190). The NFκB transcription factors are important for processes such as the regulation of the immune and inflammatory responses, development and differentiation, malignant transformation and apoptosis (191-195).

Protein kinase C
Activation of PKC is involved in lipogenesis (196), c-Fos expression (197) and regulating intracellular Ca^{2+} levels (198,199). Phospholipase C (PLC) hydrolyzes inositol phospholipids to produce inositol phosphates and 1,2-diacylglycerol (DAG), which activates PKC (40). GH-induced transcription of the spi2.1 gene requires intracellular calcium (200).

Regulation of GHR signalling

Regulation of GHR signalling by phosphatases and phosphorylation events
The most effective regulators of cytokine receptor signalling are specific phosphatases. SH2 domain-containing protein tyrosine phosphatases (SHP) 1 and 2 are both negative regulators of GHR signalling. SHP1 binds and dephosphorylates Jak2 (201,202). SHP1 knockout mice are still capable of inhibiting Jak2 activity, indicating involvement of other phosphatases (202). In addition, SHP1 is not expressed in all GHR-expressing tissues (203).

SHP2, a SHP1 homologue, is also a negative regulator of GHR signalling and is ubiquitously expressed. Downregulation of GHR signalling by SHP2 happens either directly by binding to a phosphorylated tyrosine in the GHR (204) or indirectly via signal-regulatory protein (SIRP)α1. SIRPα1 is phosphorylated after GH stimulation and induces binding of SHP2 molecule(s) (205). The interaction between the two proteins results in increased phosphatase activity of SHP2 (206). As a consequence, SIRPα1, Jak2 and possibly the GHR are dephosphorylated (207).
Receptor–like PTP subtypes H1, 1B, and CD45 can act on GHR and/or Jak2. PTP-H1 has been shown to dephosphorylate GHR in cellular models (208) and accordingly, PTPH1 knockout mice display enhanced growth (209). PTP-1B has been shown to interact with Jak2 upon GH stimulation and dephosphorylate tyrosines of activated Jak2. In addition, PTP1B binds to the phosphorylated GHR and induces its dephosphorylation. PTP1B knockout mice indeed show hyperphosphorylated Jak2 and STAT5 upon GH treatment (208-210). CD45, a phosphatase, directly binds and dephosphorylates Jaks, resulting in the negative regulation of cytokine signalling. Disruption of the CD45 gene results in enhanced Jak and STAT activation (211).
Serine phosphorylation, possibly performed by ERK1 and/or 2, results in inhibition of Jak2 kinase activity (212).
Tyrosine phosphorylation of tyrosine 119 in Jak2 results in dissociation of Jak2 from cytokine receptors, thereby abrogating Jak2 kinase activity (155).

Suppressors of cytokine signalling (SOCS)
SOCS proteins are a second class of negative regulators of the Jak/STAT pathway in cytokine signalling. To date, at least eight SOCS proteins are known, including SOCS1 to 7 and cytokine-inducible SH2 protein (CIS). All SOCS proteins have similar structures, which are featured by a central SH2 domain and a conserved C-terminal SOCS box (213). SOCS1 and 3 have an additional kinase inhibitory region (KIR) at the N-terminus (214). Furthermore, the SOCS proteins have been identified as a new class of ubiquitin ligases. They associate with cullin5-Rbx2 via a PLXP motif at the N-terminus of the SOCS box (215,216).
GH induces the expression of four SOCS proteins in several tissues. SOCS1, -3 and CIS are rapidly, but transiently upregulated, whereas the SOCS2 level gradually increases upon GH stimulation (217-219). *In vitro* overexpression studies with SOCS1 and SOCS3 showed complete inhibition of GH-dependent signalling, whereas the inhibitory effects of SOCS2 and CIS were only partial (217,220,221). All SOCS proteins affect GH signalling by differential mechanisms.
The SH2 domain of SOCS1 binds directly to tyrosine 1007 within the activation loop of Jak2 and inhibits Jak2 kinase activity through the KIR motif (222-224). This interaction results in reduced Jak2 and STAT5 phosphorylation. SOCS1 regulates proteasomal degradation of activated Jak2, probably via its ubiquitin ligase activity (225). The physiological relevance of SOCS1 in the regulation of GH signalling is unclear, since several reports have shown that in liver, which is a key tissue for GH actions, the transcriptional activation of SOCS1 is weak or not detected (217,226,227).
In order to inhibit STAT5 activation, SOCS3 binds directly to phosporylated tyrosines 333, 338 and 487. Since these tyrosine residues are probably not the major STAT5 binding sites, it might be that SOCS3 affects Jak2 (220,221). Like SOCS1, SOCS3 is able to inhibit Jak2

kinase activity through its KIR motif, although it has been shown for gp130 that SOCS3 prefers binding to the cytokine receptor instead of Jak2 (214,228). Similar to SOCS1, the physiological relevance of SOCS3 is not clear in GH signalling. Liver-specific SOCS3 knockout mice grow as wild type mice, indicating that SOCS3 does not have a major role in the regulation of body growth (229).
CIS has been proposed to act by the competitive masking of STAT5 binding sites in the activated receptor (230). In addition, CIS might promote GHR/Jak2 proteasomal degradation (230,231), since proteasome inhibition reverses the CIS effect on GH signalling without affecting CIS levels. CIS overexpression in mice has a similar phenotype as STAT5 knockout mice, indicating its role in GH signalling. However, CIS knockout mice do not have a growth phenotype, suggesting CIS redundancy (232).
Several studies in cells and mice on SOCS2 have shown that SOCS2 inhibits GH-induced STAT5 activation. SOCS2 binding to phosphorylated tyrosines 487 and 595 in the rabbit GHR suggests competition with STAT5B binding, resulting in inhibition of STAT5B activation.

Other GHR signalling regulators
Protein inhibitors of activated STATs (PIAS) proteins, which display SUMO ligase activity, bind several STAT proteins and presumably prevent DNA association (209,233).
Upon GH stimulation, GHR and adapter protein Gbr10 interact, which enables Grb10 to inhibit transcription of genes containing the serum response element of c-fos and the GH response element 2 of the Spi2.1 gene (234).
SH2Bβ has been shown to be a potent activator of Jak2. Overexpression of SH2Bβ results in the enhancement of GH-induced STAT3 and STAT5B phosphorylation (235), whereas mutation of a critical lysine in SH2Bβ disables SH2Bβ to activate Jak2 and inhibits STAT5B migration to the nucleus (236).

Cytokine receptor trafficking

Generally, type I cytokine receptors are synthesized and glycosylated in the ER and transported via the Golgi to the plasma membrane. At the plasma membrane, the receptor can perform its main function, which is the initiation of signal transduction pathways upon ligand binding. The number of receptors at the cell surface determines the responsiveness of cells for a certain ligand.
The steady state level of a receptor is the result of synthesis and degradation. Until recently, the regulation of synthesis was supposed to be the dominant factor in this equation. However, it has become clear that the degradation of signalling receptors is at least as important. Inducing or inhibiting degradation of receptors is a rapid way to regulate the responsiveness of cells towards their ligands. Cytokine receptors are selected for endocytosis at the cell surface followed by sorting to the endosomal/lysosomal system and ultimately in lysosomal degradation. To control the degradation rate of signalling receptors, nature has devised an array of pathways. Endocytosis of cytokine receptors is dependent on clathrin-coated pits. Cargo selection for clathrin-mediated endocytosis involves lots of sorting signals, which can be recognised by endocytic adapter proteins. The prototypical and best-understood adapter protein for clathrin-mediated endocytosis is Adaptor protein (AP) 2, which is, besides clathrin, an important constituent of

clathrin-coated vesicles. Sorting signals that are recognised by AP-2 are the YXXΦ (X, any amino acid; Φ, bulky hydrophobic amino acid) and the dileucine motif. These sorting signals are structurally different and recognise a different AP-2 subunit. As a consequence, the two sorting signals do not compete with each other for entry into the clathrin-coated pit. A family of endocytic adapter proteins is clathrin-associated sorting proteins (CLASPS), which binds to both AP-2 and clathrin through short peptide interaction motifs. The phosphotyrosine binding (PTB) domain-containing CLASPS recognise [FY]XNPX[YF] sorting signals, which are normally not recognised by AP-2. Ubiquitin-selective CLASPs, like EPS15 and epsin are recruited via their ubiquitin-interacting motifs (UIMs) to active receptors through monoubiquitination of receptors likely followed by the recruitment of AP-2 and clathrin at the plasma membrane (237). In our studies we identified the ubiquitination system and Jak2 as important factors in GHR activity and degradation. In this section, I will summarize the current knowledge about the role of Jak2 and the ubiquitin proteasome system in the regulation of the levels of type I cytokine receptors at the cell surface.

The role of Jak2 in cytokine receptor trafficking

The first study that suggested a role for Jak for cytokine receptors besides the activation of signalling pathways upon cytokine stimulation, describes that Tyk-/- cells displayed a reduced interferon-alpha receptor 1 (IFNAR1) protein level (238). A few years later, a role for Jak2 was implied in the Golgi processing and cell surface expression of the EpoR (166). In this study, the authors found that the N-terminal domain of Jak2 binds the immature EpoR in the ER, stimulates its folding and promotes EpoR cell surface expression. Moreover, Jak1 was shown to regulate Oncostatin M receptor (OSMR) cell surface expression (239). Finally, Jak2 and Tyk2 affect TpoR cell surface expression and stability by stimulating recycling and enhancing the protein stability of the mature, Golgi-processed form of the TpoR, whereas TpoR internalization kinetics were unaffected (240).

The role of Jak2 in GHR trafficking

For the GHR, it has been shown that Jak2 enhances the stability of the mature GHR (241) both by the stimulation of efficient processing from the ER (242) and by preventing GHR degradation (243). The authors of these studies, all performed by the same group, propose that the GHR can be degraded constitutively and GH-dependent and that the mechanisms of these pathways are different.

The ubiquitin proteasome system and receptor trafficking

Ubiquitination of a protein affects protein stability, protein interactions, enzymatic activity and localization (244-246). Especially monoubiquitination and K63-linked polyubiquitination have been associated with roles in protein trafficking (244,245,247,248). The first evidence for the role of ubiquitination in transmembrane receptor trafficking came from studies in yeast. It was found that ubiquitin is required for receptor internalisation as well as sorting receptors to the vacuole (249,250). Monoubiquitination of several transmembrane receptors (α-factor receptors, permeases and transporters) is sufficient for cargo selection at the plasma membrane, but K63-linked polyubiquitination accelerates the process (251). In mammalian systems, the GHR was the first mammalian receptor reported to depend on ubiquitination for degradation (252).

Ubiquitin ligases are the substrate-recognising factors of the ubiquitin proteasome system and determine which receptor is internalised and through which route. This makes them very important regulators of endocytosis and imply that the regulation of the interaction between an ubiquitin ligase and its substrate is essential for proper receptor trafficking. The substrate specificity of the E3 ligases, which bind in most cases directly to their targets, is usually very high. However, some interactions between E3 ligases and substrates need cofactor, adapter proteins, inhibitors or posttranslational modifications (253).
The cargo selection of ubiquitinated receptors by ubiquitin binding domain(UBD)-containing proteins has to be very precise in order to prevent missorting. The affinity of UBDs is usually low for mono- or poly-ubiquitin (254,255). Therefore, this affinity is usually increased by binding of the UBD-domain containing proteins to several monoubiquitins in multi-monoubiquitinated cargo or the binding of several UBD containing proteins to one monoubiquitin (256,257). In addition, endocytic adapters can bind at the same time to ubiquitin and to the plasma membrane. For example, epsins have a domain that recognises phosphoinositides (245).
After cargo selection at the cell surface, the next sorting step takes place at the early endosome, in which nonubiquitinated proteins are sorted to the recycling pathway back to plasma membrane or sorted to other cellular compartments. However, ubiquitinated proteins end up in MVBs, which are intraluminal vesicles (258,259). Endosomal sorting complexes required for transport (ESCRTs) are responsible for targeting ubiquitinated proteins to the MVBs. Four ESCRT complexes (0, I, II and III) are known and required to target cargo to MVBs and eventually to lysosomes (260).
In the remainder of the section, I will discuss the role of ubiquitination in the trafficking of signalling transmembrane receptors from different classes.

Epidermal growth factor receptor (EGFR)
EGFR is a receptor tyrosine kinase and its internalisation has been extensively studied. Together with Platelet-derived growth factor receptor (PDGFR), EGFR was the first receptor found to be ubiquitinated (261,262). The regulation of ligand-induced EGFR internalisation and subsequent degradation is a well-characterized mechanism. The EGFR is both mono- and polyubiquitinated upon EGF stimulation with an important role for the E3, Cbl (263). Multiple direct and indirect Cbl binding sites are present in the EGFR tail, which might facilitate the ubiquitin ligase binding. A single ubiquitin on the EGFR is already sufficient for EGFR internalisation, although a K63-linked polyubiquitin chain renders the process more efficient (264).
The EGFR internalises both via clathrin-dependent and independent endocytosis. At low EGF concentrations, the EGFR is not ubiquitinated and enters the cell via the clathrin-dependent route. In contrast, high EGF concentrations result in EGFR ubiquitination and internalisation via clathrin-independent endocytosis (265,266). It has been shown that the clathrin-dependent pathway of endocytosis sorts the EGFRs to the recycling pathway, whereas the clathrin-independent pathway mainly targets the EGFRs for degradation. These findings demonstrate how ubiquitination can determine the signalling outcome of a receptor. Strong induction of ubiquitination results in desensitisation for EGF, whereas no ubiquitination results in an increase of sensitivity by increasing the number of receptors at the cell surface via the recycling pathway (267).

After internalization, EGFR complexes can be rapidly returned to the cell surface via the recycling pathway or remain in endosomes and end up in MVBs. In the MVBs, ubiquitinated EGFR is recognised by the ESCRT-0 complex in the MVBs, which later fuse with primary lysosomes, resulting in degradation of EGF and EGFR (268).

Met receptor
Hepatocyte Growth factor (HGF) stimulation of the Met receptor results in receptor polyubiquitination and degradation, for which Cbl recruitment to phosphorylated tyrosine 1003 is essential (269,270). Degradation and polyubiquitination is dependent on Met tyrosine kinase activity (271).

β2-adrenergic receptor (β2-AR)
β2-AR is the best-studied member of the G-protein coupled receptors (GPCR), which is the largest receptor family known in mammals. Ligand binding to the β2-AR results in β2-AR phosphorylation by GRK2, which recruits the adapter protein β-arrestin to the receptor, leading to ubiquitination of both β2-AR and β-arrestin (272). β-arrestin ubiquitination is essential for β2-AR internalisation and is performed by the ubiquitin ligase Mdm2 (best known for its role in p53 degradation), which interacts with β-arrestin. β2-AR ubiquitination is not required for internalisation, but is essential for targeting for degradation. Recently, two DUBs, USP20 and USP33, has been shown to reverse β2-AR ubiquitination and its lysosomal degradation (273). USP33 deubiquitinates β-arrestin as well, resulting in stabilisation of the β2-AR-β-arrestin complex (274).

TGF-β receptor
Members of the TGF-β receptor family consists of type I and type II serine/threonine kinase receptors. Type II receptors phosphorylate type I receptors upon ligand binding. Receptor Smads 2 and 3, critical mediators of TGF-β signalling, are phosphorylated by activated type I receptors, associate with Smad4 and move to the nucleus to induce transcription of target genes, until inhibitory Smads 6 and 7 terminate the signal (275,276). The Smurf ubiquitin ligases play a key role in TGF-β signalling by targeting the receptor Smads for degradation (277,278) or by targeting TGF-β itself for degradation following association with an inhibitory Smad (279).

Tyrosine kinase receptor A (TrkA)
TrkA is, together with its coreceptor p75 neurotrophin receptor, the receptor for nerve growth factor (NGF). TrkA receptor ubiquitination is required for endocytosis and signalling (280,281). Two different hypotheses have been postulated for the mechanism of ubiquitination. The first one suggested that the p75 coreceptor and its interacting ubiquitin ligase TRAF6 is responsible for K63-linked ubiquitination of the TrkA receptor (281), whereas the second one proposes that Nedd4-2 acts in multi-monoubiquitination of TrkA (280).

EpoR
The first study that associated the EpoR with the ubiquitin proteasome system described receptor-associated ubiquitin ligase (RUL) as a ubiquitin ligase in mediating EpoR ubiquitination. Furthermore, RUL inhibited the expression of c-myc and bcl-2, two

immediate-early genes associated with Epo-induced cell growth. In addition, mutant RUL inhibited Epo-dependent cell proliferation and survival (282).
Strong evidence for the role of ubiquitination in EpoR trafficking came from a study in which was shown that, upon EpoR ubiquitination, part of the intracellular domain is removed by the proteasome. Subsequently, the remaining part of the ligand-bound EpoR is degraded by the lysosome (283). Recently, the EpoR has been shown to be dependent on the E3 ligase β-TrCP for EpoR ubiquitination and degradation. β-TrCP binds to the consensus motif DSGxxS in EpoR, of which both serines need to be phosphorylated. A disease called familial polycythemia displays hypersensitivity to Epo. The fact that patients have an alanine on the position of the first serine of the DSGxxS motif might explain this phenomenon (284).

PrlR
Prolactin stimulation results in PRLR phosphorylation, including a serine in the consensus motif for β-TrCP, which is recruited to the PRLR. β-TrCP targets the PRLR for ubiquitination and degradation (285). It has been shown that Jak2 kinase activity is required for β-TrCP action (286). Although monoubiquitination, K48-linked and K63-linked ubiquitination of the PRLR was found, only the K63-linked chains were important for PRLR endocytosis, sorting and degradation (287).

The ubiquitin system and GHR trafficking

The first indication for a role of the ubiquitin proteasome system in GHR trafficking came from an experiment in chinese hamster lung cells with a temperature sensitive mutation in the ubiquitin-activating enzyme E1. GH uptake by exogenous GHR in these cells was inhibited at the cell surface at non-permissive temperature, indicating that an intact ubiquitin system is important for GHR endocytosis. Moreover, polyubiquitination of the GHR was completely inhibited at non-permissive temperature (252). Further experimental evidence demonstrated that GHR ubiquitination, the recruitment of GHR into clathrin-coated pits and GH-induced GHR internalisation go hand in hand (57,288). Moreover, proteasome activity is required for GHR internalisation (289). It has been shown that ubiquitination of the GHR is not required for GHR endocytosis, demonstrated by the finding that a mutant GHR truncation in which all remaining lysines are mutated into alanines is still dependent on the ubiquitin system for its endocytosis.
An important finding was the identification of the Ubiquitin-dependent Endocytosis (UbE) motif, which regulates GHR endocytosis ubiquitin-system dependently. The 10-amino acid sequence of this motif is DSWVEFIELD (49). Interestingly, a di-leucine motif, present at amino acid positions 347 and 348 in the GHR, is not active in the full length GHR, but in a GHR truncated at position 349, the dileucine motif is activated. Importantly, internalisation via the dileucine motif is independent of the ubiquitin system, requires clathrin and results in GHR degradation in the lysosome (50). Recent evidence shows that the GHR can pass the ESCRT system in MVBs only if it has an intact UbE-motif (290).

Outline of this thesis

Phosphorylation and ubiquitination are important to regulate the function of proteins by affecting their activity, stability and localisation. In this thesis, we identified a ubiquitin ligase ($SCF^{\beta TrCP2}$) and a kinase (Jak2) as key players in GHR endocytosis and signal transduction.

In chapter II of this thesis, we describe how the E3 ligase $SCF^{\beta TrCP2}$ binds to the UbE-motif of the GHR and selects the GHR for endocytosis. Depletion of the F-box protein βTrCP resulted in strong inhibition of GHR endocytosis, demonstrating an essential role for this protein in GHR endocytosis. Coprecipitation of Skp1 with GHR and the requirement for neddylation for efficient GHR endocytosis revealed that βTrCP is active within a SCF complex. In addition, we found that βTrCP binds to the GHR via a non-conventional motif (UbE) rather than via the consensus βTrCP destruction motif DSGRTS, located more downstream in the GHR. Although GHR ubiquitination itself is not required for GHR endocytosis, we showed that βTrCP is involved in ubiquitination of GHR via K48 linkages (Chapter III). Moreover, in chapter II and IV, we identified the splice variant βTrCP2γ as the (likely) functional F-box protein involved in GHR endocytosis. We found that the localisation of βTrCP2 splice variants were strikingly different, which contributes to the understanding of substrate specificity of βTrCP isoforms and splice variants.

The second key player that we describe to have a critical role in GHR endocytosis is Jak2. We found that Jak2 binding to the GHR inhibits its endocytosis. Jak2 acts upstream of $SCF^{\beta TrCP}$, since GHRs in complex with Jak2 accumulated on the cell surface in a non-ubiquitinated state. In addition, we showed that GH stimulation dissociates Jak2 from the GHR, rendering GHR capable of being selected for endocytosis by $SCF^{\beta TrCP}$. Finally, we showed that both GH-induced and constitutive GHR endocytosis share a similar mechanism of endocytosis.

Jak2 has been previously described to be essential for the activation of GHR signal transduction routes such as STAT5B, MAPK and PI3-kinase. We identified the important tyrosines for GHR and STAT5B phosphorylation. In addition, we identified a specific tyrosine residue (Y627) in the GHR that could be involved in recruiting a member of the receptor–like phosphotyrosine phosphatases (PTP) family. Mutation of this tyrosine results in strong constitutive phosphorylation of the GHR, Jak2 and MAPK (Chapter V).

Finally, previous findings in combination with the data that are shown in this thesis have made us propose a model of GHR endocytosis and signalling. The findings of this thesis have contributed substantially to the understanding of GHR endocytosis and signalling and will be important for regulation of the number of GHRs at the cell surface, e.g. in cancer and cachexia patients.

References

1. Goffin, V., Shiverick, K. T., Kelly, P. A., and Martial, J. A. (1996) *Endocr Rev* **17**, 385-410
2. Mott, H. R., and Campbell, I. D. (1995) *Curr Opin Struct Biol* **5**, 114-121
3. Longhi, S. A., Cortes, M. M., and Retegui, L. A. (2003) *Growth Horm IGF Res* **13**, 353-360
4. Abdel-Meguid, S. S., Shieh, H. S., Smith, W. W., Dayringer, H. E., Violand, B. N., and Bentle, L. A. (1987) *Proc Natl Acad Sci U S A* **84**, 6434-6437
5. Kopchick, J. J. (2003) *Horm Res* **60 Suppl 3**, 103-112
6. Besson, A., Salemi, S., Deladoey, J., Vuissoz, J. M., Eble, A., Bidlingmaier, M., Burgi, S., Honegger, U., Fluck, C., and Mullis, P. E. (2005) *J Clin Endocrinol Metab* **90**, 2493-2499
7. Okada, S., and Kopchick, J. J. (2001) *Trends Mol Med* **7**, 126-132.
8. Mol, J. A., van Garderen, E., Rutteman, G. R., and Rijnberk, A. (1996) *J Steroid Biochem Mol Biol* **57**, 67-71
9. Scheepens, A., Moderscheim, T. A., and Gluckman, P. D. (2005) *Horm Res* **64 Suppl 3**, 66-72
10. Harvey, S., Kakebeeke, M., and Sanders, E. J. (2004) *J Mol Neurosci* **22**, 139-145
11. Beyea, J. A., Olson, D. M., Vandergriend, R. A., and Harvey, S. (2005) *Cell Tissue Res* **322**, 379-392
12. Kirpensteijn, J., Timmermans-Sprang, E. P., van Garderen, E., Rutteman, G. R., Lantinga-van Leeuwen, I. S., and Mol, J. A. (2002) *Mol Cell Endocrinol* **197**, 179-185
13. Kelley, K. W., Weigent, D. A., and Kooijman, R. (2007) *Brain Behav Immun* **21**, 384-392
14. Lantinga van Leeuwen, I. S., Teske, E., van Garderen, E., and Mol, J. A. (2000) *Anticancer Res* **20**, 2371-2376
15. Shuto, Y., Shibasaki, T., Otagiri, A., Kuriyama, H., Ohata, H., Tamura, H., Kamegai, J., Sugihara, H., Oikawa, S., and Wakabayashi, I. (2002) *J Clin Invest* **109**, 1429-1436
16. Rosicka, M., Krsek, M., Jarkovska, Z., Marek, J., and Schreiber, V. (2002) *Physiol Res* **51**, 435-441
17. Jarkovska, Z., Krsek, M., Rosicka, M., and Marek, J. (2004) *Endocr Regul* **38**, 80-86
18. van Buul-Offers, S. C., and Kooijman, R. (1998) *Cell Mol Life Sci* **54**, 1083-1094
19. Fruchtman, S., Jackson, L., and Borski, R. (2000) *Endocrinology* **141**, 2886-2894
20. Jansson, J. O., Eden, S., and Isaksson, O. (1985) *Endocr Rev* **6**, 128-150
21. Eden, S. (1979) *Endocrinology* **105**, 555-560
22. Tannenbaum, G. S., Choi, H. K., Gurd, W., and Waxman, D. J. (2001) *Endocrinology* **142**, 4599-4606
23. Gebert, C. A., Park, S. H., and Waxman, D. J. (1999) *Mol. Endocrinol.* **13**, 38-56.
24. Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., and Wood, W. I. (1987) *Nature* **330**, 537-543
25. Baumann, G., Stolar, M. W., Amburn, K., Barsano, C. P., and DeVries, B. C. (1986) *J Clin Endocrinol Metab* **62**, 134-141.
26. Herington, A. C., Ymer, S., and Stevenson, J. (1986) *J Clin Invest* **77**, 1817-1823
27. Veldhuis, J. D., Johnson, M. L., Faunt, L. M., Mercado, M., and Baumann, G. (1993) *J Clin Invest* **91**, 629-641
28. Baumann, G., Amburn, K., and Shaw, M. A. (1988) *Endocrinology* **122**, 976-984
29. Ayuk, J., and Sheppard, M. C. (2006) *Postgrad Med J* **82**, 24-30
30. Moller, N., and Jorgensen, J. O. (2009) *Endocr Rev* **30**, 152-177
31. Laron, Z., Pertzelan, A., and Mannheimer, S. (1966) *Isr J Med Sci* **2**, 152-155
32. Rosenfeld, R. G., Rosenbloom, A. L., and Guevara-Aguirre, J. (1994) *Endocr Rev* **15**, 369-390
33. Daughaday, W. H., and Trivedi, B. (1987) *Proc Natl Acad Sci U S A* **84**, 4636-4640
34. Grotzinger, J. (2002) *Biochim Biophys Acta* **1592**, 215-223
35. Moore, K. W., de Waal Malefyt, R., Coffman, R. L., and O'Garra, A. (2001) *Annu Rev Immunol* **19**, 683-765
36. Postel-Vinay, M. C., and Finidori, J. (1995) *Eur J Endocrinol* **133**, 654-659
37. Frank, S. J., Gilliland, G., Kraft, A. S., and Arnold, C. S. (1994) *Endocrinology* **135**, 2228-2239
38. Sotiropoulos, A., Perrot Applanat, M., Dinerstein, H., Pallier, A., Postel Vinay, M. C., Finidori, J., and Kelly, P. A. (1994) *Endocrinology* **135**, 1292-1298
39. Carter-Su, C., Schwartz, J., and Smit, L. S. (1996) *Annu Rev Physiol* **58**, 187-207.
40. Argetsinger, L. S., and Carter Su, C. (1996) *Physiol. Rev.* **76**, 1089-1107
41. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., and

Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11349-11353
42. Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6934-6938.
43. Kelly, P. A., Ali, S., Rozakis, M., Goujon, L., Nagano, M., Pellegrini, I., Gould, D., Djiane, J., Edery, M., Finidori, J., and Postel-Vinay, M. C. (1993) *Recent. Prog. Horm. Res.* **48**, 123-164
44. Kelly, P. A., Djiane, J., Postel-Vinay, M. C., and Edery, M. (1991) *Endocr Rev* **12**, 235-251
45. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255**, 306-312.
46. Fuh, G., Mulkerrin, M. G., Bass, S., McFarland, N., Brochier, M., Bourell, J. H., and Light, D. W., J.A. (1990) *J. Biol. Chem.* **265,**, 3111-3115.
47. Baumgartner, J. W., Wells, C. A., Chen, C. M., and Waters, M. J. (1994) *J. Biol. Chem.* **269,**, 29094-29101.
48. VanderKuur, J. A., Wang, X., Zhang, L., Campbell, G. S., Allevato, G., Bellestrup, N., Norstedt, G., and Carter-Su, C. (1994) *J. Biol. Chem.* **269**, 21709-21717.
49. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) *EMBO J.* **18**, 28-36.
50. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1998) *J. Biol. Chem.* **273**, 16426-16433
51. Gent, J., van Kerkhof, P., Roza, M., Bu, G., and Strous, G. J. (2002) *Proc Nat Acad Sci USA* **99**, 9858-9863
52. Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) *Nat Struct Mol Biol* **12**, 814-821
53. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinology* **143**, 1243-1252
54. Strous, G. J., van Kerkhof, P., Govers, R., Rotwein, P., and Schwartz, A. L. (1997) *J. Biol. Chem.* **272**, 40-43
55. Alves dos Santos, C. M., ten Broeke, T., and Strous, G. J. (2001) *J Biol Chem* **276**, 32635-32641.
56. Baumann, G. (1994) *J. Endocrinol.* **141**, 1-6
57. van Kerkhof, P., Sachse, M., Klumperman, J., and Strous, G. J. (2000) *J. Biol. Chem.* **276**, 3778-3784.
58. Sachse, M., van Kerkhof, P., Strous, G. J., and Klumperman, J. (2001) *J Cell Sci* **114**, 3943-3952
59. van Kerkhof, P., and Strous, G. J. (2001) *Biochem Soc Trans* **29**, 488-493.
60. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67**, 425-479
61. Wilkinson, K. D. (2005) *PNAS* **102**, 15280-15282
62. Li, W., and Ye, Y. (2008) *Cell Mol Life Sci* **65**, 2397-2406
63. Vijay-Kumar, S., Bugg, C. E., and Cook, W. J. (1987) *J Mol Biol* **194**, 531-544
64. Grabbe, C., and Dikic, I. (2009) *Chem Rev* **109**, 1481-1494
65. Sun, L., and Chen, Z. J. (2004) *Curr Opin Cell Biol* **16**, 119-126
66. Pickart, C. M. (2001) *Annu Rev Biochem* **70**, 503-533.
67. Frescas, D., and Pagano, M. (2008) *Nat Rev Cancer* **8**, 438-449
68. Hoeller, D., Hecker, C. M., Wagner, S., Rogov, V., Dotsch, V., and Dikic, I. (2007) *Mol Cell* **26**, 891-898
69. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999) *Cell* **96**, 635-644
70. Schulman, B. A., and Harper, J. W. (2009) *Nat Rev Mol Cell Biol* **10**, 319-331
71. Patton, E. E., Willems, A. R., and Tyers, M. (1998) *Trends in Genetics* **14**, 236-243
72. Deshaies, R. J. (1999) *Annu Rev Cell Dev Biol* **15**, 435-467
73. Koepp, D. M., Harper, J. W., and Elledge, S. J. (1999) *Cell* **97**, 431-434
74. Tyers, M., and Jorgensen, P. (2000) *Curr Opin Genet Dev* **10**, 54-64
75. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) *Cell* **82**, 915-925
76. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996) *Cell* **86**, 263-274
77. Connelly, C., and Hieter, P. (1996) *Cell* **86**, 275-285
78. Barral, Y., Jentsch, S., and Mann, C. (1995) *Genes Dev* **9**, 399-409
79. Schwob, E., Bohm, T., Mendenhall, M. D., and Nasmyth, K. (1994) *Cell* **79**, 233-244
80. Willems, A. R., Schwab, M., and Tyers, M. (2004) *Biochim Biophys Acta* **1695**, 133-170
81. Orlicky, S., Tang, X., Willems, A., Tyers, M., and Sicheri, F. (2003) *Cell* **112**, 243-256
82. Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B., and Goebl, M. G. (1996) *Mol Cell Biol* **16**, 6634-6643
83. Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W., and Hedgecock, E. M. (1996) *Cell* **85**, 829-839
84. Zachariae, W., Shevchenko, A., Andrews, P. D., Ciosk, R., Galova, M., Stark, M. J., Mann, M., and

Nasmyth, K. (1998) *Science* **279**, 1216-1219
85. Dias, D. C., Dolios, G., Wang, R., and Pan, Z. Q. (2002) *Proc Natl Acad Sci U S A* **99**, 16601-16606
86. Nikolaev, A. Y., and Gu, W. (2003) *Cell Cycle* **2**, 169-171
87. Merlet, J., Burger, J., Gomes, J. E., and Pintard, L. (2009) *Cell Mol Life Sci* **66**, 1924-1938
88. Angers, S., Li, T., Yi, X., Maccoss, M. J., Moon, R. T., and Zheng, N. (2006) *Nature*
89. Goldenberg, S. J., Cascio, T. C., Shumway, S. D., Garbutt, K. C., Liu, J., Xiong, Y., and Zheng, N. (2004) *Cell* **119**, 517-528
90. Duda, D. M., Borg, L. A., Scott, D. C., Hunt, H. W., Hammel, M., and Schulman, B. A. (2008) *Cell* **134**, 995-1006
91. Hao, B., Oehlmann, S., Sowa, M. E., Harper, J. W., and Pavletich, N. P. (2007) *Mol Cell* **26**, 131-143
92. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) *Mol Cell* **11**, 1445-1456
93. Xirodimas, D. P. (2008) *Biochem Soc Trans* **36**, 802-806
94. Rabut, G., and Peter, M. (2008) *EMBO Rep* **9**, 969-976
95. Pan, Z. Q., Kentsis, A., Dias, D. C., Yamoah, K., and Wu, K. (2004) *Oncogene* **23**, 1985-1997
96. Saifee, N. H., and Zheng, N. (2008) *Cell* **135**, 209-211
97. Wu, K., Chen, A., and Pan, Z. Q. (2000) *Journal of Biological Chemistry* **275**, 32317-32324
98. Sakata, E., Yamaguchi, Y., Miyauchi, Y., Iwai, K., Chiba, T., Saeki, Y., Matsuda, N., Tanaka, K., and Kato, K. (2007) *Nat Struct Mol Biol* **14**, 167-168
99. Saha, A., and Deshaies, R. J. (2008) *Mol Cell* **32**, 21-31
100. Petroski, M. D., and Deshaies, R. J. (2005) *Cell* **123**, 1107-1120
101. Tang, O. S., Lin Z, Willems A, Neculai D, Ceccarelli D, Mercurio F, Shilton BH, Sicheri F, Tyers M. (2007) *Cell* **129**, 1165-1176
102. Kleiger, G., Saha, A., Lewis, S., Kuhlman, B., and Deshaies, R. J. (2009) *Cell* **139**, 957-968
103. Bornstein, G., Ganoth, D., and Hershko, A. (2006) *PNAS* **103**, 11515-11520
104. Sufan, R. I., and Ohh, M. (2006) *Neoplasia* **8**, 956-963
105. Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A. F., Tanaka, K., and Nakatani, Y. (2003) *Cell* **113**, 357-367
106. Barbash, O., Zamfirova, P., Lin, D. I., Chen, X., Yang, K., Nakagawa, H., Lu, F., Rustgi, A. K., and Diehl, J. A. (2008) *Cancer Cell* **14**, 68-78
107. Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998) *Mol Cell* **1**, 565-574
108. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23**, 2028-2036
109. Coadou, G., Gharbi-Benarous, J., Megy, S., Bertho, G., Evrard-Todeschi, N., Segeral, E., Benarous, R., and Girault, J. P. (2003) *Biochemistry* **42**, 14741-14751
110. Nakayama, K. I., and Nakayama, K. (2005) *Semin Cell Dev Biol* **16**, 323-333
111. Karin, M., and Ben-Neriah, Y. (2000) *Annu Rev Immunol* **18**, 621-663
112. Joyce, D., Albanese, C., Steer, J., Fu, M., Bouzahzah, B., and Pestell, R. G. (2001) *Cytokine Growth Factor Rev* **12**, 73-90
113. Fuchs, S. Y., Chen, A., Xiong, Y., Pan, Z. Q., and Ronai, Z. (1999) *Oncogene* **18**, 2039-2046
114. Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., and Polakis, P. (1999) *Curr Biol* **9**, 207-210
115. Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K., and Nakayama, K. (1999) *Embo Journal* **18**, 2401-2410
116. Maniatis, T. (1999) *Genes & Development* **13**, 505-510
117. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev.* **13**, 270-283
118. Wilkinson, K. D. (1997) *FASEB J* **11**, 1245-1256
119. Baker, R. T., and Board, P. G. (1987) *Nucleic Acids Res* **15**, 443-463
120. Ozkaynak, E., Finley, D., Solomon, M. J., and Varshavsky, A. (1987) *EMBO J* **6**, 1429-1439
121. Wiborg, O., Pedersen, M. S., Wind, A., Berglund, L. E., Marcker, K. A., and Vuust, J. (1985) *EMBO J* **4**, 755-759
122. Reyes-Turcu, F. E., Ventii, K. H., and Wilkinson, K. D. (2009) *Annu Rev Biochem* **78**, 363-397
123. Pickart, C. M., and Rose, I. A. (1985) *J Biol Chem* **260**, 7903-7910

124. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) *Cell* **123**, 773-786
125. Wilkinson, K. D., Tashayev, V. L., O'Connor, L. B., Larsen, C. N., Kasperek, E., and Pickart, C. M. (1995) *Biochemistry* **34**, 14535-14546
126. Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K. D., Cohen, R. E., and Pickart, C. M. (1997) *J Biol Chem* **272**, 23712-23721
127. Bignell, G. R., Warren, W., Seal, S., Takahashi, M., Rapley, E., Barfoot, R., Green, H., Brown, C., Biggs, P. J., Lakhani, S. R., Jones, C., Hansen, J., Blair, E., Hofmann, B., Siebert, R., Turner, G., Evans, D. G., Schrander-Stumpel, C., Beemer, F. A., van Den Ouweland, A., Halley, D., Delpech, B., Cleveland, M. G., Leigh, I., Leisti, J., and Rasmussen, S. (2000) *Nat Genet* **25**, 160-165
128. Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Kessler, B. M. (2002) *Chem Biol* **9**, 1149-1159
129. Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D., and Courtois, G. (2003) *Nature* **424**, 801-805
130. Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A., and Mosialos, G. (2003) *Nature* **424**, 793-796
131. Geiss-Friedlander, R., and Melchior, F. (2007) *Nat Rev Mol Cell Biol* **8**, 947-956
132. Yamoah, K., Oashi, T., Sarikas, A., Gazdoiu, S., Osman, R., and Pan, Z. Q. (2008) *Proc Natl Acad Sci U S A* **105**, 12230-12235
133. Petroski, M. D. (2008) *BMC Biochem* **9 Suppl 1**, S7
134. Hall, J. M., Lee, M. K., Newman, B., Morrow, J. E., Anderson, L. A., Huey, B., and King, M. C. (1990) *Science* **250**, 1684-1689
135. Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., Miki, Y., and et al. (1994) *Science* **266**, 120-122
136. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., and et al. (1994) *Science* **266**, 66-71
137. Brzovic, P. S., Meza, J., King, M. C., and Klevit, R. E. (1998) *J Biol Chem* **273**, 7795-7799.
138. Brzovic, P. S., Meza, J. E., King, M. C., and Klevit, R. E. (2001) *J Biol Chem* **28**, 28
139. Brzovic, P. S., Rajagopal, P., Hoyt, D. W., King, M. C., and Klevit, R. E. (2001) *Nat Struct Biol* **8**, 833-837
140. Davies, J. E., Sarkar, S., and Rubinsztein, D. C. (2007) *BMC Biochem* **8 Suppl 1**, S2
141. Rui, L., Yuan, M., Frantz, D., Shoelson, S., and White, M. F. (2002) *J Biol Chem* **277**, 42394-42398
142. Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K., Pan, Z.Q., Valenzuela, D. M., DeChiara, T. M., Stitt, T. N., Yancopoulos, G. D., and Glass, D. J. (2001) *Science* **249**, 1704-1708
143. Waters, M. J., Hoang, H. N., Fairlie, D. P., Pelekanos, R. A., and Brown, R. J. (2006) *J Mol Endocrinol* **36**, 1-7
144. Biener-Ramanujan, E., Ramanujan, V. K., Herman, B., and Gertler, A. (2006) *Growth Hormone & IGF Research* **16**, 247-257
145. Barclay, J. L., Kerr, L. M., Arthur, L., Rowland, J. E., Nelson, C. N., Ishikawa, M., d'Aniello, E. M., White, M., Noakes, P. G., and Waters, M. J. (2009) *Mol Endocrinol*
146. Yamaoka, K., Saharinen, P., Pesu, M., Holt, V. E., 3rd, Silvennoinen, O., and O'Shea, J. J. (2004) *Genome Biol* **5**, 253
147. Musso, T., Johnston, J. A., Linnekin, D., Varesio, L., Rowe, T. K., O'Shea, J. J., and McVicar, D. W. (1995) *J Exp Med* **181**, 1425-1431
148. Tortolani, P. J., Lal, B. K., Riva, A., Johnston, J. A., Chen, Y. Q., Reaman, G. H., Beckwith, M., Longo, D., Ortaldo, J. R., Bhatia, K., McGrath, I., Kehrl, J., Tuscano, J., McVicar, D. W., and O'Shea, J. J. (1995) *J Immunol* **155**, 5220-5226
149. Behrmann, I., Smyczek, T., Heinrich, P. C., Schmitz-Van de Leur, H., Komyod, W., Giese, B., Muller-Newen, G., Haan, S., and Haan, C. (2004) *J. Biol. Chem.* **279**, 35486-35493
150. Rodig, S. J., Meraz, M. A., White, J. M., Lampe, P. A., Riley, J. K., Arthur, C. D., King, K. L., Sheehan, K. C., Yin, L., Pennica, D., Johnson, E. M., Jr., and Schreiber, R. D. (1998) *Cell* **93**, 373-383
151. Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998) *Cell* **93**, 397-409
152. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93**, 385-395

153. Macchi, P., Villa, A., Giliani, S., Sacco, M. G., Frattini, A., Porta, F., Ugazio, A. G., and e.a. Notarangelo, L. D. (1995) *Nature* **377,**, 65-68.
154. Russell, S. M., Tayebi, N., Nakajima, H., Riedy, M. C., Roberts, J. L., Aman, M. J., Migone, T. S., Noguchi, M., Markert, M. L., Buckley, R. H., O'Shea, J. J., and Leonard, W. J. (1995) *Science* **270**, 797-800
155. Funakoshi-Tago, M., Pelletier, S., Matsuda, T., Parganas, E., and Ihle, J. N. (2006) *Embo J* **25**, 4763-4772
156. Robertson, S. A., Koleva, R. I., Argetsinger, L. S., Carter-Su, C., Marto, J. A., Feener, E. P., and Myers, M. G., Jr. (2009) *Mol Cell Biol* **29**, 3367-3378
157. Argetsinger, L. S., Kouadio, J. L., Steen, H., Stensballe, A., Jensen, O. N., and Carter-Su, C. (2004) *Mol Cell Biol* **24**, 4955-4967
158. Feener, E. P., Rosario, F., Dunn, S. L., Stancheva, Z., and Myers, M. G., Jr. (2004) *Mol Cell Biol* **24**, 4968-4978
159. Kurzer, J. H., Argetsinger, L. S., Zhou, Y. J., Kouadio, J. L., O'Shea, J. J., and Carter-Su, C. (2004) *Mol Cell Biol* **24**, 4557-4570
160. Feng, J., Witthuhn, B. A., Matsuda, T., Kohlhuber, F., Kerr, I. M., and Ihle, J. N. (1997) *Mol Cell Biol* **17**, 2497-2501
161. Funakoshi-Tago, M., Tago, K., Kasahara, T., Parganas, E., and Ihle, J. N. (2008) *Cell Signal* **20**, 1995-2001
162. Saharinen, P., Takaluoma, K., and Silvennoinen, O. (2000) *Mol Cell Biol* **20**, 3387-3395
163. Saharinen, P., Vihinen, M., and Silvennoinen, O. (2003) *Mol Biol Cell* **14**, 1448-1459
164. Zhao, L., Dong, H., Zhang, C. C., Kinch, L., Osawa, M., Iacovino, M., Grishin, N. V., Kyba, M., and Huang, L. J. (2009) *J Biol Chem* **284**, 26988-26998
165. Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. (1992) *Cell* **70**, 313-322
166. Huang, L. J., Constantinescu, S. N., and Lodish, H. F. (2001) *Mol Cell* **8**, 1327-1338
167. Zhou, Y. J., Chen, M., Cusack, N. A., Kimmel, L. H., Magnuson, K. S., Boyd, J. G., Lin, W., Roberts, J. L., Lengi, A., Buckley, R. H., Geahlen, R. L., Candotti, F., Gadina, M., Changelian, P. S., and O'Shea, J. J. (2001) *Mol Cell* **8**, 959-969
168. Barua, D., Faeder, J. R., and Haugh, J. M. (2009) *PLoS Comput Biol* **5**, e1000364
169. Rosenfeld, R. G., Belgorosky, A., Camacho-Hubner, C., Savage, M. O., Wit, J. M., and Hwa, V. (2007) *Trends Endocrinol Metab* **18**, 134-141
170. Levy, D. E., and Darnell, J. E., Jr. (2002) *Nat Rev Mol Cell Biol* **3**, 651-662
171. O'Sullivan, L. A., Liongue, C., Lewis, R. S., Stephenson, S. E., and Ward, A. C. (2007) *Mol Immunol* **44**, 2497-2506
172. Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997) *Proc Natl Acad Sci U S A* **94**, 3801-3804
173. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) *Cell* **84**, 443-450
174. Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and Schreiber, R. D. (1996) *Cell* **84**, 431-442
175. Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997) *Genes Dev* **11**, 179-186
176. Teglund, S., McKay, C., Schuetz, E., van Deursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93**, 841-850
177. Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S. H., Ram, P. A., Waxman, D. J., and Davey, H. W. (1997) *Proc Natl Acad Sci U S A* **94**, 7239-7244.
178. Hansen, L. H., Wang, X. Z., Kopchick, J. J., Bouchelouche, P., Nielsen, J. H., and Billestrup, N. (1996) *J. Biol. Chem.* **271**, 12669-12673
179. VanderKuur, J. A., Wang, X. Y., Zhang, L. Y., Allevato, G., Billestrup, N., and CarterSu, C. (1995) *J. Biol. Chem.* **270,**, 21738-21744.
180. Cobb, M. H., and Goldsmith, E. J. (1995) *J Biol Chem* **270**, 14843-14846
181. Zhang, W., and Liu, H. T. (2002) *Cell Res* **12**, 9-18
182. VanderKuur, J. A., Butch, E. R., Waters, S. B., Pessin, J. E., Guan, K. L., and CarterSu, C. (1997) *Endocrinology* **138**, 4301-4307.

183. Zhu, T., Goh, E. L., and Lobie, P. E. (1998) *J Biol Chem* **273**, 10682-10689.
184. Zhu, T., and Lobie, P. E. (2000) *J Biol Chem* **275**, 2103-2114.
185. Yamauchi, T., Kaburagi, Y., Ueki, K., Tsuji, Y., Stark, G. R., Kerr, I. M., Tsushima, T., Akanuma, Y., Komuro, I., Tobe, K., Yazaki, Y., and Kadowaki, T. (1998) *Journal of Biological Chemistry* **273**, 15719-15726
186. Moutoussamy, S., Kelly, P. A., and Finidori, J. (1998) *Eur J Biochem* **255**, 1-11.
187. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14**, 4902-4911.
188. Songyang, Z., Baltimore, D., Cantley, L. C., Kaplan, D. R., and Franke, T. F. (1997) *Proc Natl Acad Sci U S A* **94**, 11345-11350.
189. Jeay, S., Sonenshein, G. E., Postel-Vinay, M. C., Kelly, P. A., and Baixeras, E. (2002) *Mol Cell Endocrinol* **188**, 1-7
190. Jeay, S., Sonenshein, G. E., Kelly, P. A., Postel-Vinay, M. C., and Baixeras, E. (2001) *Endocrinology* **142**, 147-156
191. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87**, 13-20
192. Baldwin, A. S., Jr. (1996) *Annu Rev Immunol* **14**, 649-683
193. Barnes, P. J., and Karin, M. (1997) *N Engl J Med* **336**, 1066-1071
194. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu Rev Immunol* **16**, 225-260
195. Foo, S. Y., and Nolan, G. P. (1999) *Trends Genet* **15**, 229-235
196. Smal, J., and De Meyts, P. (1987) *Biochem Biophys Res Commun* **147**, 1232-1240
197. Slootweg, M. C., De Groot, R. P., Herrmann-Erlee, M. P., Koornneef, I., Kruijer, W., and Kramer, Y. M. (1991) *J Mol Endocrinol* **6**, 179-188
198. Gaur, S., Yamaguchi, H., and Goodman, H. M. (1996) *Am J Physiol* **270**, C1478-C1484
199. Gaur, S., Yamaguchi, H., and Goodman, H. M. (1996) *Am J Physiol* **270**, C1485-C1492
200. Billestrup, N., Bouchelouche, P., Allevato, G., Ilondo, M., and Nielsen, J. H. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2725-2729
201. Jiao, H., Berrada, Yang, W., Tabrizi, M., Platanias, L. C., and Yi, T. (1996) *Mol Cell Biol* **16**, 6985-6992
202. Hackett, R. H., Wang, Y. D., Sweitzer, S., Feldman, G., Wood, W. I., and Larner, A. C. (1997) *J. Biol. Chem.* **272**, 11128-11132
203. Yi, T., Cleveland, J., and Ihle, J. (1992) *Mol. Cell. Biol.* **12**, 836-846
204. Stofega, M. R., Herrington, J., Billestrup, N., and Carter-Su, C. (2000) *Mol Endocrinol* **14**, 1338-1350
205. Stofega, M. R., Wang, H., Ullrich, A., and Carter-Su, C. (1998) *J Biol Chem* **273**, 7112-7117
206. Ohnishi, H., Kubota, M., Ohtake, A., Sato, K., and Sano, S. (1996) *J Biol Chem* **271**, 25569-25574
207. Stofega, M. R., Argetsinger, L. S., Wang, H., Ullrich, A., and Carter-Su, C. (2000) *J Biol Chem* **275**, 28222-28229
208. Pasquali, C., Curchod, M. L., Walchli, S., Espanel, X., Guerrier, M., Arigoni, F., Strous, G., and Hooft Van Huijsduijnen, R. (2003) *Mol Endocrinol* **17**, 2228-2239
209. Pilecka, I., Whatmore, A., Hooft van Huijsduijnen, R., Destenaves, B., and Clayton, P. (2007) *Trends Endocrinol Metab* **18**, 12-18
210. Gu, F., Dube, N., Kim, J. W., Cheng, A., Ibarra-Sanchez Md Mde, J., Tremblay, M. L., and Boisclair, Y. R. (2003) *Mol Cell Biol* **23**, 3753-3762
211. Irie-Sasaki, J., Sasaki, T., Matsumoto, W., Opavsky, A., Cheng, M., Welstead, G., Griffiths, E., Krawczyk, C., Richardson, C. D., Aitken, K., Iscove, N., Koretzky, G., Johnson, P., Liu, P., Rothstein, D. M., and Penninger, J. M. (2001) *Nature* **409**, 349-354.
212. Mazurkiewicz-Munoz, A. M., Argetsinger, L. S., Kouadio, J. L., Stensballe, A., Jensen, O. N., Cline, J. M., and Carter-Su, C. (2006) *Mol Cell Biol* **26**, 4052-4062
213. Wormald, S., and Hilton, D. J. (2004) *J. Biol. Chem.* **279**, 821-824
214. Sasaki, A., Yasukawa, H., Suzuki, A., Kamizono, S., Syoda, T., Kinjyo, I., Sasaki, M., Johnston, J. A., and Yoshimura, A. (1999) *Genes Cells* **4**, 339-351
215. Kamura, T., Burian, D., Yan, Q., Schmidt, S. L., Lane, W. S., Querido, E., Branton, P. E., Shilatifard, A., Conaway, R. C., and Conaway, J. W. (2001) *J Biol Chem* **276**, 29748–29753
216. Kamura, T., Maenaka, K., Kotoshiba, S., Matsumoto, M., Kohda, D., Conaway, R. C., Conaway, J. W., and Nakayama, K. I. (2004) *Genes Dev* **18**, 3055-3065
217. Adams, T. E., Hansen, J. A., Starr, R., Nicola, N. A., Hilton, D. J., and Billestrup, N. (1998) *J. Biol. Chem.*

273, 1285-1287
218. Paul, C., Seiliez, I., Thissen, J. P., and Le Cam, A. (2000) *Eur J Biochem* **267**, 5849-5857
219. Tollet-Egnell, P., Floresmorales, A., Stavreusevers, A., Sahlin, L., and Norstedt, G. (1999) *Endocrinology* **140**, 3693-3704
220. Hansen, J. A., Lindberg, K., Hilton, D. J., Nielsen, J. H., and Billestrup, N. (1999) *Mol. Endocrinol.* **13**, 1832-1843
221. Ram, P. A., and Waxman, D. J. (1999) *J. Biol. Chem.* **274**, 35553-35561
222. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., and Yoshimura, A. (1997) *Nature* **387**, 921-924
223. Nicholson, S. E., Willson, T. A., Farley, A., Starr, R., Zhang, J. G., Baca, M., Alexander, W. S., Metcalf, D., Hilton, D. J., and Nicola, N. A. (1999) *EMBO J* **18**, 375-385
224. Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N., and Yoshimura, A. (1999) *Embo J.* **18**, 1309-1320.
225. Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D. J., and Silvennoinen, O. (2002) *Mol Cell Biol* **22**, 3316-3326
226. Davey, H. W., Park, S. H., Grattan, D. R., McLachlan, M. J., and Waxman, D. J. (1999) *J Biol Chem* **274**, 35331-35336
227. Boisclair, Y. R., Wang, J., Shi, J., Hurst, K. R., and Ooi, G. T. (2000) *J Biol Chem* **275**, 3841-3847
228. Nicholson, S. E., De Souza, D., Fabri, L. J., Corbin, J., Willson, T. A., Zhang, J. G., Silva, A., Asimakis, M., Farley, A., Nash, A. D., Metcalf, D., Hilton, D. J., Nicola, N. A., and Baca, M. (2000) *Proc Natl Acad Sci U S A* **97**, 6493-6498
229. Croker, B. A., Krebs, D. L., Zhang, J. G., Wormald, S., Willson, T. A., Stanley, E. G., Robb, L., Greenhalgh, C. J., Forster, I., Clausen, B. E., Nicola, N. A., Metcalf, D., Hilton, D. J., Roberts, A. W., and Alexander, W. S. (2003) *Nat Immunol* **4**, 540-545
230. Ram, P. A., and Waxman, D. J. (2000) *J. Biol. Chem.* **275**, 39487-39496.
231. Du, L., Frick, G. P., Tai, L.-R., Yoshimura, A., and Goodman, H. M. (2003) *Endocrinology* **144**, 868-876
232. Matsumoto, A., Seki, Y., Kubo, M., Ohtsuka, S., Suzuki, A., Hayashi, I., Tsuji, K., Nakahata, T., Okabe, M., Yamada, S., and Yoshimura, A. (1999) *Mol Cell Biol* **19**, 6396-6407
233. Rakesh, K., and Agrawal, D. K. (2005) *Biochem Pharmacol* **70**, 649-657
234. Moutoussamy, S., Renaudie, F., Lago, F., Kelly, P. A., and Finidori, J. (1998) *J Biol Chem* **273**, 15906-15912.
235. Rui, L., and Carter-Su, C. (1999) *Proc Natl Acad Sci U S A* **96**, 7172-7177
236. Herrington, J., Diakonova, M., Rui, L., Gunter, D. R., and CarterSu, C. (2000) *Journal of Biological Chemistry* **275**, 13126-13133
237. Traub, L. M. (2009) *Nat Rev Mol Cell Biol* **10**, 583-596
238. Gauzzi, M. C., Barbieri, G., Richter, M. F., Uze, G., Ling, L., Fellous, M., and Pellegrini, S. (1997) *Proc Natl Acad Sci U S A* **94**, 11839-11844
239. Radtke, S., Hermanns, H. M., Haan, C., Schmitz-Van De Leur, H., Gascan, H., Heinrich, P. C., and Behrmann, I. (2002) *J Biol Chem* **277**, 11297-11305
240. Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J., and Constantinescu, S. N. (2005) *J. Biol. Chem.* **280**, 27251-27261
241. He, K., Loesch, K., Cowan, J. W., Li, X., Deng, L., Wang, X., Jiang, J., and Frank, S. J. (2005) *Endocrinology* **146**, 4755-4765
242. Loesch, K., Deng, L., Wang, X., He, K., Jiang, J., and Frank, S. J. (2007) *Endocrinology* **148**, 5955-5965
243. Deng, L., He, K., Wang, X., Yang, N., Thangavel, C., Jiang, J., Fuchs, S. Y., and Frank, S. J. (2007) *Mol Endocrinol* **21**, 1537-1551
244. Salmena, L., and Pandolfi, P. P. (2007) *Nat Rev Cancer* **7**, 409-413
245. Mukhopadhyay, D., and Riezman, H. (2007) *Science* **315**, 201-205
246. Weissman, A. M. (2001) *Nat Rev Mol Cell Biol* **2**, 169-178.
247. Pickart, C. M., and Fushman, D. (2004) *Curr Opin Chem Biol* **8**, 610-616
248. Huang, T. T., and D'Andrea, A. D. (2006) *Nat Rev Mol Cell Biol* **7**, 323-334
249. Hicke, L., and Riezman, H. (1996) *Cell* **84**, 277-287
250. Kölling, R., and Hollenberg, C. P. (1994) *EMBO J.* **13**, 3261-3271

251. Galan, J. M., and Haguenauer-Tsapis, R. (1997) *EMBO Journal* **16**, 5847-5854
252. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) *EMBO J.* **15**, 3806-3812
253. Leon, S., and Haguenauer-Tsapis, R. (2009) *Exp Cell Res* **315**, 1574-1583
254. Hurley, J. H., Lee, S., and Prag, G. (2006) *Biochem J* **399**, 361-372
255. Hicke, L., Schubert, H. L., and Hill, C. P. (2005) *Nat Rev Mol Cell Biol* **6**, 610-621
256. Lee, S., Tsai, Y. C., Mattera, R., Smith, W. J., Kostelansky, M. S., Weissman, A. M., Bonifacino, J. S., and Hurley, J. H. (2006) *Nat Struct Mol Biol*
257. Penengo, L., Mapelli, M., Murachelli, A. G., Confalonieri, S., Magri, L., Musacchio, A., Di Fiore, P. P., Polo, S., and Schneider, T. R. (2006) *Cell* **124**, 1183-1195
258. Hicke, L., and Dunn, R. (2003) *Annu Rev Cell Dev Biol* **19**, 141-172
259. Piper, R. C., and Luzio, J. P. (2007) *Curr Opin Cell Biol* **19**, 459-465
260. Williams, R. L., and Urbe, S. (2007) *Nat Rev Mol Cell Biol* **8**, 355-368
261. GalchevaGargova, Z., Theroux, S. J., and Davis, R. J. (1995) *Oncogene* **11**, 2649-2655
262. Mori, S., Heldin, C. H., and Claesson-Welsh, L. (1992) *J. Biol. Chem.* **267,**, 6429-6434.
263. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) *Mol Cell* **21**, 737-748
264. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) *Nat Cell Biol* **5**, 461-466
265. Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P., and Polo, S. (2005) *PNAS* **102**, 2760-2765
266. Woelk, T., Oldrini, B., Maspero, E., Confalonieri, S., Cavallaro, E., Di Fiore, P. P., and Polo, S. (2006) *Nat Cell Biol* **8**, 1246-1254
267. Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., and Di Fiore, P. P. (2008) *Dev Cell* **15**, 209-219
268. Sorkin, A., and Goh, L. K. (2008) *Exp Cell Res* **314**, 3093-3106
269. Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S., and Park, M. (2005) *Mol. Cell. Biol.* **25**, 9632-9645
270. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. (2001) *Mol Cell* **8**, 995-1004
271. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vandewoude, G. F. (1997) *Mol Cell Biol.* **17**, 799-808
272. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) *Science* **294**, 1307-1313
273. Berthouze, M., Venkataramanan, V., Li, Y., and Shenoy, S. K. (2009) *EMBO J* **28**, 1684-1696
274. Shenoy, S. K., Modi, A. S., Shukla, A. K., Xiao, K., Berthouze, M., Ahn, S., Wilkinson, K. D., Miller, W. E., and Lefkowitz, R. J. (2009) *Proc Natl Acad Sci U S A* **106**, 6650-6655
275. Massague, J., and Chen, Y. G. (2000) *Genes Dev* **14**, 627-644
276. Mehra, A., and Wrana, J. L. (2002) *Biochem Cell Biol* **80**, 605-622
277. Lin, X., Liang, M., and Feng, X. H. (2000) *J Biol Chem* **275**, 36818-36822
278. Zhang, Y., Chang, C., Gehling, D. J., Hemmati-Brivanlou, A., and Derynck, R. (2001) *Proc Natl Acad Sci U S A* **98**, 974-979
279. Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) *Mol Cell* **6**, 1365-1375.
280. Arevalo, J. C., Waite, J., Rajagopal, R., Beyna, M., Chen, Z. Y., Lee, F. S., and Chao, M. V. (2006) *Neuron* **50**, 549-559
281. Geetha, T., Jiang, J., and Wooten, M. W. (2005) *Mol Cell* **20**, 301-312
282. Friedman, A. D., Nimbalkar, D., and Quelle, F. W. (2003) *J Biol Chem* **278**, 26851–26861
283. Walrafen, P., Verdier, F., Kadri, Z., Chretien, S., Lacombe, C., and Mayeux, P. (2005) *Blood* **105**, 600-608
284. Meyer, L., Deau, B., Forejtnikova, H., Dumenil, D., Margottin-Goguet, F., Lacombe, C., Mayeux, P., and Verdier, F. (2007) *Blood*
285. Li, Y., Suresh Kumar, K. G., Tang, W., Spiegelman, V. S., and Fuchs, S. Y. (2004) *Mol. Cell. Biol.* **24**, 4038-4048
286. Swaminathan, G., Varghese, B., Thangavel, C., Carbone, C. J., Plotnikov, A., Kumar, K. G., Jablonski, E. M., Clevenger, C. V., Goffin, V., Deng, L., Frank, S. J., and Fuchs, S. Y. (2008) *J Endocrinol* **196**, R1-7

287. Varghese, B., Barriere, H., Carbone, C. J., Banerjee, A., Swaminathan, G., Plotnikov, A., Xu, P., Peng, J., Goffin, V., Lukacs, G. L., and Fuchs, S. Y. (2008) *Mol Cell Biol* **28**, 5275-5287
288. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) *EMBO J.* **16**, 4851-4858
289. van Kerkhof, P., Govers, R., Alves dosSantos, C. M. A., and Strous, G. J. (2000) *J Biol Chem* **275**, 1575-1580
290. van Kerkhof, P., Putters, J., and Strous, G. J. (2007) *J Biol Chem* **282**, 20475-20483

CHAPTER II

The Ubiquitin ligase SCF(βTrCP) regulates the degradation of the growth hormone receptor

Peter van Kerkhof, Joyce Putters and Ger J. Strous

Department of Cell Biology and Institute of Biomembranes
University Medical Center Utrecht

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Abstract

SCF ubiquitin ligases play a pivotal role in the regulation of cell division and various signal transduction pathways which in turn are involved in cell growth, survival and transformation. SCF(βTrCP) recognises the double phosphorylated DSGΦXS destruction motif in βcatenin and IκB. We show that the same ligase drives endocytosis and degradation of the growth hormone (GH) receptor in a ligand-independent fashion. The F-box protein βTrCP binds directly and specifically with its WD40 domain to a novel recognition motif, previously designated as the ubiquitin-dependent endocytosis motif. Receptor degradation requires an active neddylation system, implicating ubiquitin ligase activity. GHR-βTrCP binding, but not GHR ubiquitination, is necessary for endocytosis. βTrCP2 silencing is more effective on GHR degradation and endocytosis than βTrCP1, although overexpression of either isoform restores βTrCP function in silenced cells. Together, these findings provide direct evidence for a key role of the SCF(βTrCP) in the endocytosis and degradation of an important factor in growth, immunity and life span regulation.

Introduction

Understanding how metabolism of cells and organisms is regulated and how this connects to the regulation of longevity, cell cycle, apoptosis and immunity are major challenges of modern biology (1). Growth hormone and its receptor (GHR) act at these crossroads as becomes apparent in the current study in which we show that a regulator of cell cycle and immunity appears to regulate GH sensitivity of cells. All cells of the human body contain GHRs. Upon GH binding these receptors initiate signal transduction that results in expression of genes involved in anabolic processes including increased protein synthesis, lipid degradation, immune function, muscle mass, bone turnover, and tooth development (2). The GHR was the first class-one cytokine receptor to be cloned (3). Signal transduction of class-one cytokine receptors recruits members of the Janus kinase family tyrosine kinases that bind to a proline-rich motif in the intracellular membrane-proximal domain, the box 1 motif (4). For the GHR, Jak2 acts as initiator of several signalling cascades including Stat activation. Box 1-related activities contribute little to GHR endocytosis (5). For receptor tyrosine kinases both signal transduction and receptor abundance are regulated by their ligands: EGF, c-kit, insulin and Notch binding initiate not only signal transduction, they also induce a swift degradation of their receptors. For cytokine receptors these two parameters seem to have their own independent regulating mechanism. There is good evidence that the GH sensitivity of cells is mainly regulated via the Ubiquitin-dependent Endocytosis motif (UbE motif) in the cytokine receptor box 2 region (6). Mutation of UbE motif (DDSWVEFIELD) leads to a dramatic drop in endocytosis and lysosomal degradation, rendering the cells more GH-sensitive.

The ubiquitin-proteasome system provides the major pathway for non-lysosomal degradation, reviewed in: (7). The F-box protein β-transducing repeat-containing protein (βTrCP) serves as the substrate recognition subunit in the SCF(βTrCP) ligase (8). Classical examples of substrates are β-catenin, NFκB, and inhibitor of NFκB (IκB). Two isoforms exist: βTrCP1 is mainly present in the nucleus, while βTrCP2 resides in the cytosol (9). These ligases play a pivotal role in cell division and various essential signal transduction pathways for tumorigenesis. Their substrate binding motifs consist of seven propeller-shaped WD40 domains that specifically bind short motifs in its central cavity. These motifs generally are 6 amino acid residue-long with a common structure of DSG(X)2+nS. To be recognized by the SCF(βTrCP) both serine residues need to be phosphorylated. Recently, it became evident that, for a restricted number of plasma membrane proteins, ubiquitination triggers internalisation and vacuolar/lysosomal rather than proteasomal degradation, (reviewed in: (10). This pathway is best understood in yeast, where a number of plasma membrane proteins are endocytosed in an ubiquitin-dependent manner (11-12). Several studies have shown that mono-ubiquitination of plasma membrane proteins is sufficient to stimulate their endocytosis (11,13). In mammalian cells, the GHR, the β-adrenergic receptor, and the epithelial sodium channel, ENaC, are examples of membrane proteins that endocytose in an ubiquitin system-dependent manner (14-16). In some cytokine receptors the conserved DSG(X)2+nS motif serves as a degradation signal via the SCF(βTrCP) ligases. Both for the interferon-γ and the PrL receptor, it was shown that this E3 is involved in their degradation. An important finding in these studies was that receptor degradation mainly depends on ligand binding; in addition, phosphorylation at both serine residues of the degron was required (17-19).

In the present study we show that the UbE motif, deviant from the canonical βTrCP recognition motif, is specifically recognized by a functional SCF(βTrCP) complex and that this interaction is instrumental in the endocytosis and degradation of the GHR. Ubiquitination of the GHR is not required since βTrCP silencing slows degradation of the GHR without lysine residues in the cytosolic tail as efficient as wild type GHR.

Experimental procedures

Materials

βTrCP1 and βTrCP2 cDNA in pcDNA3 expressing the flag-tagged proteins were generous gifts of Tomoki Chiba, Tokyo Metropolitan Institute of Medical Science. Full-length rabbit GHR cDNA in pcDNA3 was described (20). GST fusion proteins expressing GST-GHR(271-334), and GST-GHR(271-318) were produced as described (21). PcDNA-GHR constructs used for alanine scanning of the UbE motif and GHR1-399,K271-362R, designated as GHR399-KallR, were constructed as described before (22). The following siRNA sense sequences were used to silence: both βTrCP1 and ⊚2: GUGGAAUUUGUGGAACAUCtt (called 'combi probe'), βTrCP1 only: GAUAAUACCAGAGAAGAAUtt, βTrCP2 only: GAGGCCAUCAGAAGGAAACtt, Clathrin Heavy Chain: GCAAUGAGCUGUUUGAAGAUU, GFP and HPV E6/E7 (used as controls): GGCUACGUCCAGGAGCGCACC, and CUAACUAACACUGGGUUAUtt, respectively. Dharmacon siGENOME SMARTpool's BTRC NM-003939 was used to silence βTrCP1, and FBXW11, NM-012300 to silence βTrCP2. QuikChange mutagenesis primers to introduce 3 silent mutations in the target sequence of βTrCP1 for combi TrCP, 5'-TGGTCAGAGTCAGATCAAGTGGAGTTCGTCGAACATCTTATAT CCCAAATGTG-3' and to introduce 3 silent mutations in the target sequence of TrCP2 for combi βTrCP, 5'-TGGTCTGAATCAGATCAAGTGGAGTTCGTCGAACATCTTATTTCA CGAATGTG-3'. Chemically synthesized siRNA duplexes were purchased from Ambion, Austin Texas. Antibody, recognizing the cytoplasmic domain of the GHR, was raised against amino acids 271—318 of the cytosolic tail of the GHR (23). Antibody (Mab5) against the extracellular domain of the GHR was from Agen, Acacia Ridge (Australia), goat anti-mouse IgG Alexa680 from Molecular Probes, and goat anti- rabbit IgG IRDye800 from Rockland Immunochemicals Inc, Gilbertsville (PA). Mouse monoclonal anti-FLAG (M2) was from Sigma. The polyclonal anti-βTrCP1 serum was raised in rabbits against a βTrCP1-peptide consisting of amino acids 1-93 fused to gluthathione S-transferase. The fusion protein was produced by cloning the DNA encoding amino acids 1-93 of TrCP1 into vector pGEX1λt, after which the construct was expressed in Escherichia Coli. Immobilized streptavidin was from Pierce, glutathione-sepharose from Amersham Biosciences, and Ni-NTA agarose from Qiagen. Culture media, fetal calf serum (FCS), L-glutamine, and antibiotics for tissue culture were purchased from In VitroGen.

Cell culture

Hek293 and ts41 cells were grown in DMEM (In VitroGen) supplemented with 10% FCS, 100 units/ml Penicillin and 0.1 mg/ml streptomycin. Hek293 cells stable expressing the wtGHR (Hek-wtGHR) were grown in the same medium supplemented 0.6 mg/ml Geneticin (G418; Gibco).All cells used were grown at 37ºC with 5.0% CO_2. Twice a week

cells were washed with phosphate buffered saline (PBS), detached from the flask with Trypsin-EDTA (In VitroGen), diluted in fresh growth medium and split into new culture flasks.

Transfections

Cells were transfected using Lipofectamin 2000 (Invitrogen). Seventy percent confluent cultures were transfected with 0.2 - 0.9 μg DNA in 12-well plates according to manufacturer's protocol. Transfected cells were used for Western blot experiments and ^{125}I-GH binding experiments 1-2 days after transfection. For fluorescence microscopy cells were transfected with Fugene-6 (Roche).

To silence the expression of both βTrCPs Hek-wtGHR cells were transfected with small interfering RNA (siRNA) using Lipofectamin-2000 according to the description of the manufacturer; 3 days after transfection cells were used for western blot and ^{125}I-GH binding experiments. Control cells were transfected with siRNA-GFP. To control the effect of the gene expression silencing Hek293 cells were transiently transfected with a pcDNA3-Flag-βTrCP. Western blots were analysed using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, Nebraska).

^{125}I-GH binding en internalisation

^{125}I-Human GH was prepared using chloramine T (20). For internalisation experiments, cells, grown in poly-D-lysine coated 12-well plates, were washed with 1 ml MEM supplemented with 20 mM Hepes, pH 7.4 and 0.1% bovine serum albumin (BSA), and incubated at 37ºC in a water bath for 1 hour. After washing the cells three times with ice-cold PBS-complete (PBS-c: PBS with 0.135g/l $CaCl_2$ and 0.1 g/l $MgCl_2$), 0.75 ml ^{125}I-GH (180 ng/ml in Mem/Hepes/0.1% BSA) was added to the cells. ^{125}I-GH was bound for 2 hours on ice. Unbound ^{125}I-GH was aspirated and the cells were washed three times with PBS-c. Cells were incubated 10 minutes at 37ºC in MEM/Hepes/0.1% BSA to allow receptor internalisation and washed three times with ice-cold PBS-c. Membrane associated ^{125}I-GH was removed by treating the cells twice with 750 μl acid wash (0.15 M NaCl, 50 mM glycine, 0.1% BSA, pH 2.5) for 5 minutes on ice. Acid wash was collected in counting tubes and radioactivity was measured using a LKB gamma counter. Cells were solubilized overnight in 1 N NaOH. Internalised ^{125}I-GH was determined by measuring the radioactivity in the collected NaOH. Unspecific counts were determined by incubating the cells with ^{125}I-GH together with excess unlabelled GH (9 μg/ml). Internalisation is expressed as a percentage of the total specific radioactivity after 10 min at 37 ºC.

In vitro binding assay

Wild-type and mutant GHR proteins were expressed in Hek293, the cells were lysed in 50 mM Tris HCl (pH 7.5), 0.15 or 1.0 M NaCl, 50 mM NaF, and 0.5% Nonidet P-40. The receptors were purified with biotinylated GH and streptavidin beads, and stringently washed with lysis buffer. The beads were incubated with in vitro-translated and ^{35}S-labelled βTrCP2 for 60 min at 4°C, extensively washed with lysis buffer, and associated proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. cDNA of βTrCP2 constructs in pcDNA3.1/FLAG was used in an in-vitro transcription translation system supplemented with [^{35}S]

methionine and TNT®—T7 coupled reticulocyte lysate ready reaction mix according to the instructions of the manufacturer (Promega). Binding to GHR1-286 was taken as background. To analyse GHR-containing protein complexes at the cell surface, the cells were incubated with biotinylated GH on ice. To recover all GHR-protein complexes from cells, the cells were first lysed and then incubated with biotinylated-GH. Equal amounts of cell lysates were incubated for 1 hour at 4°C with equal amounts of GST-GHR(271-334) or GST-GHR(271-286) bound to GSH beads. Beads were pelleted in an Eppendorf centrifuge and washed three times with 1 ml lysis buffer and twice with PBS containing protease inhibitors. The beads were boiled in 2x sample buffer. Samples were subjected to SDS PAGE. Proteins, associated with the GST-fusion proteins, were visualized by autoradiography. To analyse equal loading of the fusion proteins the gels were stained with Coomassie Brillant Blue and analysed using an Odyssey imaging system.

Production of GST-GHR fusion proteins

pGEX-GHR plasmids were used to express GST-GHR fusion proteins in Escherichia coli (strain BL21) (21). The synthesis of recombinant proteins was induced by isopropyl-1-thio-β-D-galactopyranoside. GST fusion proteins were purified with GSH-beads (Amersham Pharmacia Biotech) by the procedure recommended by the manufacturer.

Immune fluorescence microscopy

Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier's instructions (Amersham Biosciences). Transfected cells, grown on coverslips, were incubated for 15 min with Cy3-GH (1 μg/ml) and/or Alexa488-labeled transferrin (5 μg/ml). Cells were washed with PBS to remove unbound label and fixed for 2 h in 3% paraformaldehyde in PBS. After fixation, the cells were embedded in Mowiol, and confocal laser scanning microscopy was performed using a Leica TCS 4D system.

Results

Expression of βTrCP is required for GHR endocytosis

Endocytosis of the GHR depends on the ubiquitination machinery, implicating the involvement of an ubiquitin ligase. As recent studies show that βTrCP is involved in the degradation of certain cytokine receptors we used small interfering RNA to silence both βTrCP isoforms and study the effects on GHR turnover. Fig. 1A shows that siRNA for βTrCP inhibits GH uptake to the same extend as silencing of clathrin heavy chain: approximately 50% as compared to non-relevant siRNA (GFP). In this experiment, the clathrin heavy chain western blot signal was reduced to 4% compared to siRNA(GFP)-treated cells. Expression of cotransfected flag-tagged βTrCP2 was reduced to 10% using the 'combi' silencing oligonucleotides that silence both TrCP1 and TrCP2 (Fig. 1B). We conclude that βTrCP is involved in endocytosis of the GHR.

At steady state almost all (mature, 130 kDa) GHRs are present at the cell surface. To visualize the effect of βTrCP silencing on endocytosis we incubated the cells with both cy3-labelled GH and Alexa-488-labelled transferrin. Fig. 1C shows that silencing both βTrCP isoforms resulted in a strong increase of cy3GH at the plasma membrane, while, transferrin recycling remained intact, excluding a general effect on clathrin-mediated

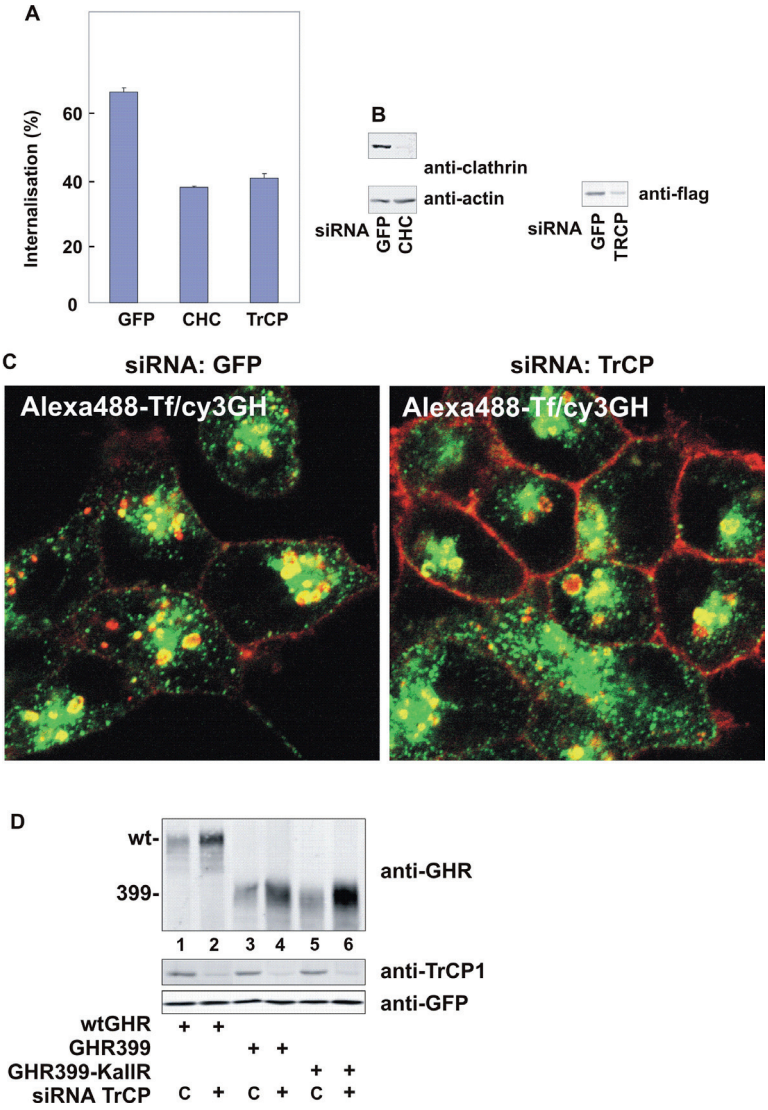

Fig. 1. Effects of TrCP on GHR endocytosis and abundance

A. GHR-expressing Hek293 cells were transfected with siRNA to silence βTrCP or clathrin heavy chain (CHC); siRNA for green fluorescent protein (GFP) was applied as a control. 125I-GH was bound to the cells on ice, the cells were incubated for 10 min at 37°C, and GH internalisation was expressed as a percentage of total radioactivity (cell surface and intracellular). B. Cell extracts from parallel experiments as in A were analysed by quantitative western blotting according to the Odyssey protocol. The efficiency of βTrCP siRNA oligonucleotides was measured on cells co-transfected with flag-TrCP2. C. GHR-expressing Hek293 cells, cultured on cover slips, were either transfected with βTrCP siRNA or with control (GFP) siRNA oligonucleotides, incubated with Alexa488-labelled transferrin (Alexa488-Tf) and cy3-labelled GH (cy3-GH) for 15 min, and fixed with formaldehyde. The confocal images show that GH and transferrin co-localised both at the cell surface and in endosomes (left panel). If TrCP siRNA oligo's were present, cy3GH (red) stained mainly the cell surface while the transferrin (green)distribution remained unaffected. Note that internalised GH only partially co-localised with transferrin, indicating sorting between the recycling transferrin and GH. D. Hek293 cells were co-transfected with GFP-DNA and different GHR constructs, and transfected with siRNA oligonucleotides as indicated. The cells were incubated with biotinylated GH for 15 min and GHRs were isolated on streptavidin beads and analysed for cell surface GHR according to the Odyssey protocol (upper panel). Equal aliquots from the cell lysates were also analysed on western blots for βTrCP1 (middle panel) and GFP (lower panel). Control siRNA (c) was HPV E6/E7. Data are representative of 3 experiments.

endocytosis. These results demonstrate that βTrCP is an obligatory part of the GHR endocytosis machinery. As both GH and transferrin are endocytosed via clathrin-coated vesicles (24) it suggests that βTrCP is part of the cargo selection complex for the GHR.

An obvious consequence of endocytosis inhibition is an accumulation of GHRs at the cell surface. In Fig. 1D, lanes 1 and 2, we used Hek293 cells transfected with GHR DNA and silenced for both βTrCP isoforms. To measure the amount of GHRs at the cell surface, the cells were incubated with biotin-GH and the GH-GHR complexes were isolated and quantitated. Si-RNA(βTrCP) treatment caused a 2-3-fold increase in GHR at the cell surface. To ascertain that the increase was not due to a silencing effect on the amount GHRs synthesized, metabolic labelling with ^{35}S-methionine was performed, and showed that the GHR synthesis was not changed (not shown). The cellular concentration of βTrCP1 was measured on western blots (Fig. 1D). By this method, using an optimized protocol for βTrCP, we obtained approximately 90% silencing. Using this silencing oligonucleotide, βTrCP2 was silenced to the same extend (Fig. 1B). Previous studies have shown that by inhibiting GHR endocytosis through (mutation of) the UbE motif maximally 60% inhibition of GHR endocytosis was achieved compared to removal of the GHR tail including the complete UbE motif. Apparently, both βTrCP and clathrin silencing were insufficient to overcome this half-maximal effect on GHR turnover. Combining the two si-RNAs did not result in more endocytosis inhibition. In agreement with our previous experience, these effects are too limited to measure half-life differences in dynamic experiments successfully, e.g. using pulse-chase protocols (21).

As an important control, based on our previous observations that the GHR itself is not an obligatory ubiquitination target, we used a GHR truncated at amino acid residue 399, in which all 10 lysines are replaced by arginine residues (GHR1–399 K271–362R) (22). Fig. 1D, lanes 3-6 shows that TrCP silencing induces a 2-3-fold increase in cell surface GHRs whether or not lysine residues are present in the receptor tail. To control the experiments of Fig. 1D for equal transfection efficiencies we co-transfected GFP DNA in all situations, and analysed the cell lysates for GFP: under all (silencing) conditions and various DNA constructs, the transfection efficiency was within measurement limits identical (Fig. 1D, lower panel).

As we previously concluded that GHR internalisation requires the recruitment of the ubiquitin conjugation system to the GHR UbE motif rather than the conjugation of ubiquitin to the GHR itself, we now conclude that βTrCP is a prime candidate to act as E3.

Other factors of the βTrCP-GHR complex

Once established that βTrCP is involved in GHR endocytosis and degradation, we asked whether βTrCP is in a protein complex with the GHR. Pull-down experiments with biotin-GH, depicted in Fig. 2A, show that both co-expressed full-length (lane 4) and mutant TrCP without the F-box domain (lane 5), were present in the isolated complexes. If His6-TrCP1 was used to isolate the protein complex (Fig. 2B), both ER and cell surface GHR were pulled down (lane 4). The interaction occurs intracellularly as shown in an experiment, in which GHR and His6-TrCP1 were expressed in different plates (lanes 1 and 5), and mixed after cell lysis (lane 3). Under these conditions (low cellular concentrations, temperature and short time periods) βTrCP does not interact with GHR. Thus, TrCP is in a protein complex with GHR, probably already in the ER.

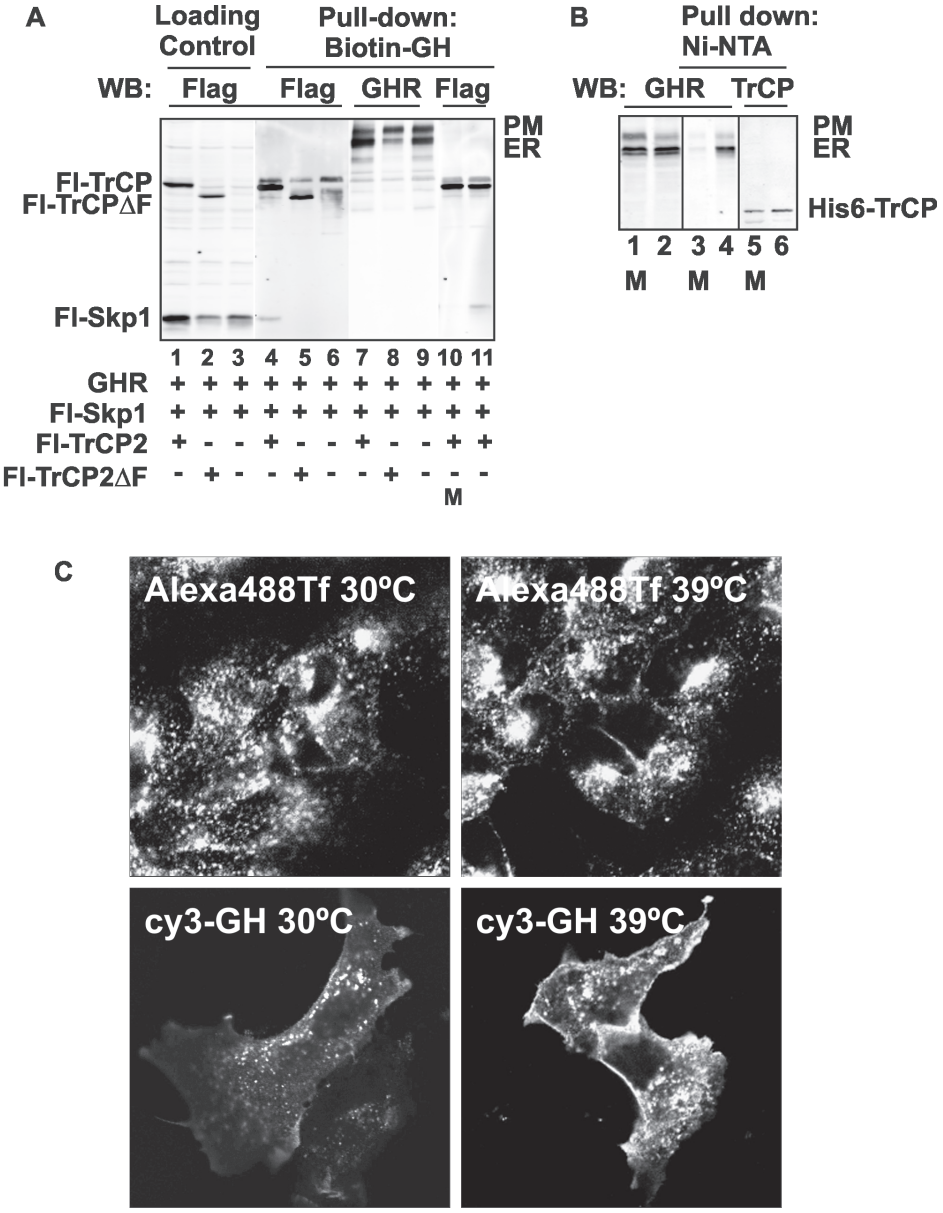

Fig. 2. SCF(TrCP) is involved in the regulation of GHR abundance

A. Hek293 cells were co-trans-fected as indicated, and lysed in 0.6 ml. 0.55 ml of the cell extracts were incubated with biotinylated GH, the protein complexes were isolated with streptavidin beads and analysed on blots using anti-GHR (mab5) or anti-flag. For loading control, 15 µl of cell lysate were used. For lane 10 ("mix" experiment), GHR/βTrCP DNAs were cotransfected in one plate of cells and GHR/Skp1 DNA in a second. Both plates were lysed in half the volume of the other samples and mixed together before the addition of biotin-GH (M). ER indicates GHR species in the endoplasmic reticulum, while PM indicates the mature GHR at the plasma membrane. Data are representative of 3 experiments. B. The lysate of Hek293 cells, co-transfected with GHR and his6-tagged βTrCP, was used to pull-down protein complexes by Ni-NTA beads. For the "mix" experiment, (M)) GHR and His6-βTrCP were transfected in separate dishes (lanes 1, 5) and the cell lysates were mixed as in the "mix" experi¬ment of Fig. 2A (lane 3). Lanes 1 and 2 are loading controls for GHR, lanes 5 and 6, for TrCP. Data are representative of 2 experiments. C. Ts-41 cells were transfected with the GHR construct, and incubated at 30°C or 39°C overnight. Cy3GH and Alexa488-transferrin were added for 15 minutes, and the cells were fixed. Data are representative of 2 experiments.

βTrCP generally acts as subunit of a SCF ubiquitin ligase. To examine the functional complex in GHR endocytosis, we co-transfected βTrCP2 and Skp1 together with the GHR. Pull-down experiments with biotinylated GH and western blot analysis of the isolated complex revealed that Skp1 interacted with GHR only in the presence of βTrCP (Fig. 2A, lane 4). In the absence of βTrCP (lane 6) or in the presence of βTrCP lacking the F-box (lane 5), no Skp1 was isolated. In lanes 10-11 we repeated the experiment and asked whether Skp1 might have associated with the GHR protein complex after lysis. As for βTrCP, the interaction does not occur if cell lysates from cells, expressing the components separately, were mixed (M). Together, this implies that GH, GHR, βTrCP2 and Skp1 are in the same protein complex.

To analyse the involvement of an active SCF complex we took a genetic approach and

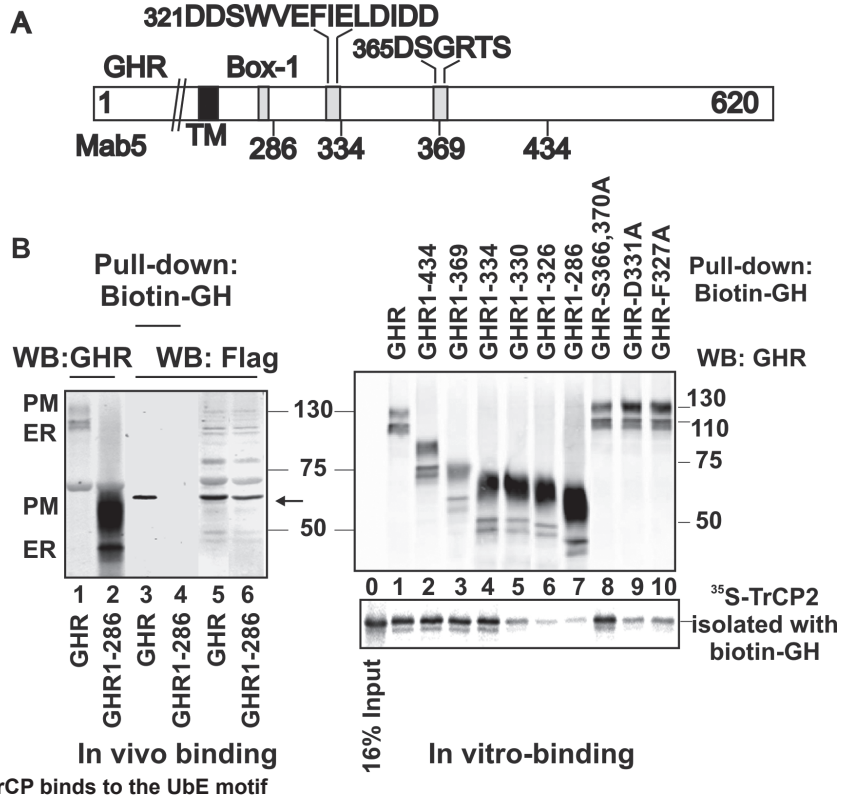

Fig. 3. βTrCP binds to the UbE motif

A. Structure of the GHR constructs. GHR contains 620 amino acids: 1-246, extracellular GH binding domain, 247-270, trans-membrane (TM) domain, and 350 amino acids in the cytosolic tail. Box 1 is the proline-rich region where Jak2 binds, and the UbE motif is between amino acid 321 and 334. GHR1-334 is truncated immediately after the UbE motif and GHR1-369 is truncated in the middle of the DSGXXS degron. B, left panel. Hek293 cells were transfected with flag-TrCP and either GHR or GHR truncated at 286 (GHR1-286). The cells were lysed and protein complexes were isolated with biotin-GH/streptavidin beads (lanes 3, 4) and analysed on blot using anti-flag. Lanes 1, 2 loading control; GHR (mab5), lanes 5, 6 loading control βTrCP (arrow). B, right panel. Ten 6-cm plates of Hek293 cells were transfected with equal amounts of DNA expressing 10 different GHR constructs as indicated. The cell lysates were incubated with biotin-GH, the receptors were isolated on streptavidin beads and the beads were washed in 0.5 M NaCl in lysis buffer; 10% of the beads were boiled in SDS-sample buffer and the samples were detected for GHR on western blot (GHR, upper panel); the remainder of the beads were incubated for 60 min on ice with equal amounts of 35S-in vitro labelled βTrCP2, washed twice and, after SDS-PAGE, the radioactivity was visualized using a Molecular Dynamics Phosphorimager. The amounts of radioactivity were determined using ImageQuant. In the left lane 16% of the input amount of radioactive βTrCP was analysed. Data are representative of 3 experiments.

expressed GHR in hamster ts41 cells. These cells contain a temperature-sensitive mutation in the NEDD8 activation enzyme, APP-BP1 and fail to degrade substrates via E3s with Cullin family members (e.g. p27 via the SCF(skp2) (25), due to defective Neddylation. Thus, the E3-SCF(TrCP) is itself regulated via covalent modification of the ubiquitin-like protein Nedd8 by UBC12, while deNeddylation is performed by the Signalosome complex. This modification stabilizes the E2-Cullin/Rbx1 interaction and in turn stimulates ubiquitination of the target substrates. Fig 2C shows that cy3-GH is endocytosed at the permissive temperature, but largely remains at the cell surface at the non-permissive temperature (39°C). In the same experiment, Alexa488-labelled transferrin entered the cells undisturbed, although there seemed to be a different distribution intracellularly. This shows that an intact Neddylation system is specifically required for uptake of the GHR. Together with the results on βTrCP and Skp1, these data strongly suggest that GHR endocytosis requires a fully functional SCF(βTrCP) complex.

βTrCP interacts with the GHR tail via the UbE motif

To further examine the role of βTrCP in GHR endocytosis, we analysed βTrCP2, as it is dominantly active in the cytosol (26). Hek293 cells were co-transfected with flag-tagged βTrCP2 together with full-length or the truncation GHR1-286 (Fig. 3A). GHR1-286 still contains box 1, the attachment site for Jak2, but lacks the UbE motif. This receptor is unable to endocytose and, therefore, accumulates at the cell surface, resulting in a high amount of mature receptor (55 kDa) compared to its 40 kD ER form (Fig. 3B, left panel, lane 2). The wild-type GHR migrates at 130 kDa, while its precursor ER form appears as a doublet at 110 kDa (lane 1). Cell lysates were incubated with biotinylated GH and GHR protein-complexes were isolated with streptavidin beads. Comparing the full-length GHR with GHR truncated at 286 shows that co-transfected flag-labelled βTrCP2 was only isolated if full-length GHR was present (Fig. 3B, left panel, lanes 3, 4). In Fig. 3B, right panel, we used various GHR truncations and mutations to pin-point the tail segment involved in βTrCP binding. The receptors were purified from transiently transfected Hek293 cells, via biotinylated GH and streptavidin beads, and then incubated on ice with equal amounts of in vitro synthesized ^{35}S-βTrCP. Wild-type GHR, GHR truncated at 434, 369 and 334 bound approximately 16% of the input ^{35}S-labelled TrCP. GHRs truncated at 330, 326, and 286, as well as the UbE mutants GHR-D331A and GHR-F327A showed little binding. Mutation of the serine residues in the DSGRTS motif of the full-length GHR (GHR-S366,370A) did not affect the GHR-SCF(TrCP) interaction (Fig. 3B, right panel, lane 8).

The interaction specificity between GHR and βTrCP2 complies with its role in endocytosis

Previously, we have shown that endocytosis of the GHR depends both on a functional ubiquitination system and on an intact UbE motif (20,27,28). Using mutational analysis of the UbE motif by changing every single amino acid residue into alanine the contribution of each amino acid was determined. The experiment identified the key residues in the UbE motif (DDSWVEFIELD) for endocytosis. As shown in Fig. 1, βTrCP is an obvious factor to act as a regulator of GHR endocytosis. Therefore, we used the pull-down assay of Fig. 3B, right panel to ask whether βTrCP binds to the GHR in a specific fashion. Each of the amino acids 320-334 were changed in alanine residues and the 15 GHR constructs were tested in Hek293 cells. The cells were transfected with equal amounts of the different constructs and showed

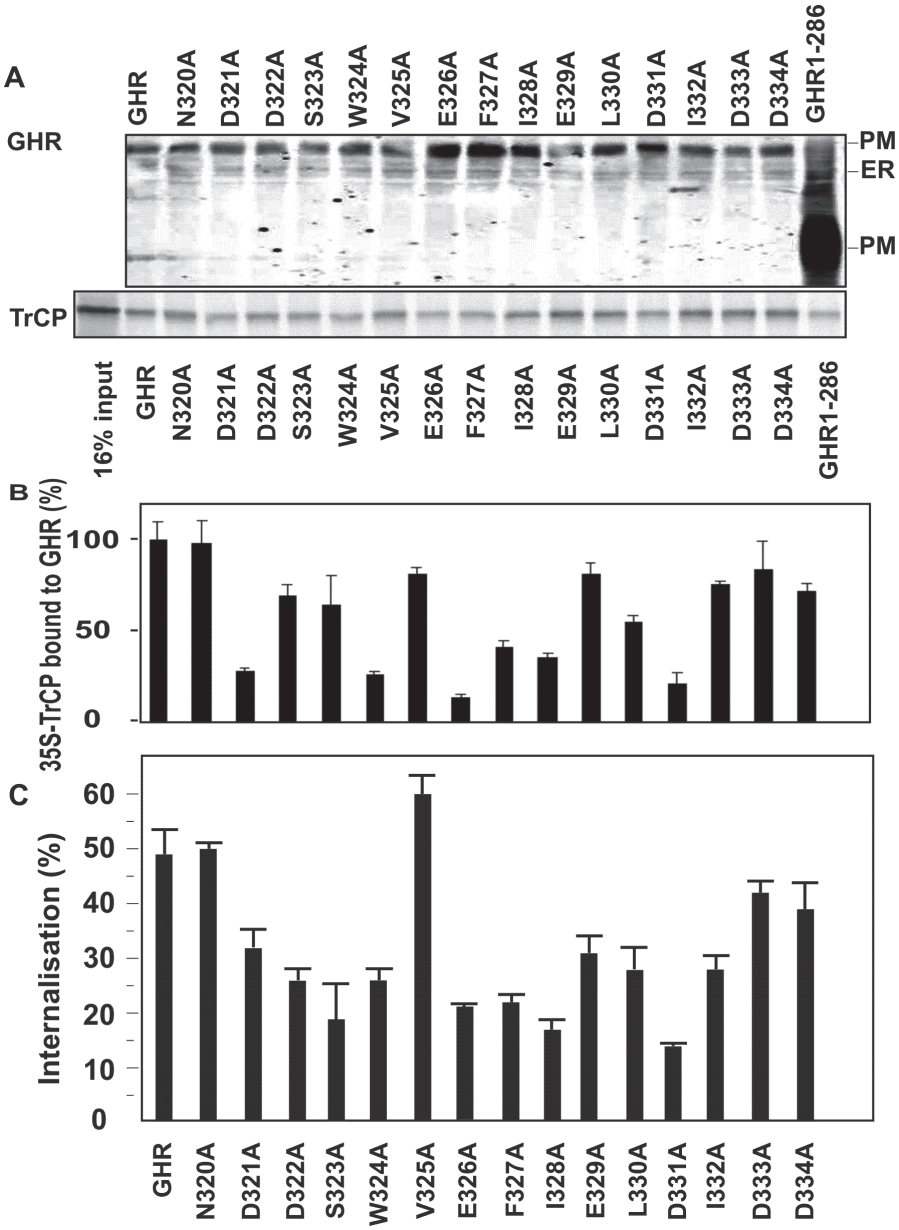

Fig. 4. Mutational analysis of the UbE motif

A. Hek293 cells were transfected with equal amounts of DNA expressing different GHR constructs as indicated. The experiment was performed as described in Fig. 3B, right panel. As controls DNA encoding wild-type GHR and GHR1-286 were used. After 24h the cells were incubated with biotin-GH on ice, lysed, and the cell surface receptors were isolated on streptavidin beads; 10% of the beads were boiled in SDS-sample buffer and the samples were detected for GHR on western blot (GHR). The remainder of the beads was incubated for 60 min on ice with 35S-in vitro labelled TrCP2. The radioactivity was visualized in lower panel (A), and quantitated in B; the amount of label co-puri¬fied with the short GHR1-286 was deduced and the results were expressed as percentages of radioactivity bound to wild type GHR. Data are representative of 2 experiments. C. Previously published data from Fig. 4 in (28). Chinese hamster Ts20 cell lines expressing wild-type and mutant GHR as indicated were analysed for rates of 125I-GH endocytosis. Internalisa¬tion is expressed as the ratio of radioactive GH present inside the cells versus at the cell surface.

comparable GHR expression judged by the amounts of high-mannose (ER-localized) GHR (not shown). Western blot analysis of the cell extracts shows that the mutations E326A, F327A, I328A, and D331A caused a significant increase in mature (130 kDa) GHR (Fig. 4A), indicative for impaired degradation. Biotin-GH was used to isolate the GH-GHR complexes from the cell surface and the GHRs were incubated with equal amounts of ^{35}S-labelled βTrCP. Most mutations resulted in inhibition of βTrCP binding as compared to wild-type GHR. However, mutation of the same 4 amino acids inhibited the βTrCP binding with more than 60% (Fig. 4B). Compared to the mutational analysis previously performed for endocytosis (Fig. 4C, and Fig. 4 in ref. 22), we note a striking similarity in patterns: the same 4 amino acid are important for endocytosis of the GHR (27).

GHR binds to the WD-40 domain of βTrCP

βTrCP canonically binds to the DSGΦXS motif in IκB, β-catenin, and HIV Vpu after both serine residues are phosphorylated. To characterize the βTrCP-GHR binding further, we used bacterially produced GST-GHR fusion proteins containing only the 65 membrane-proximal amino acid tail-residues and *in vitro* synthesized ^{35}S-βTrCP (Fig. 5A) (21). Fig. 5B,

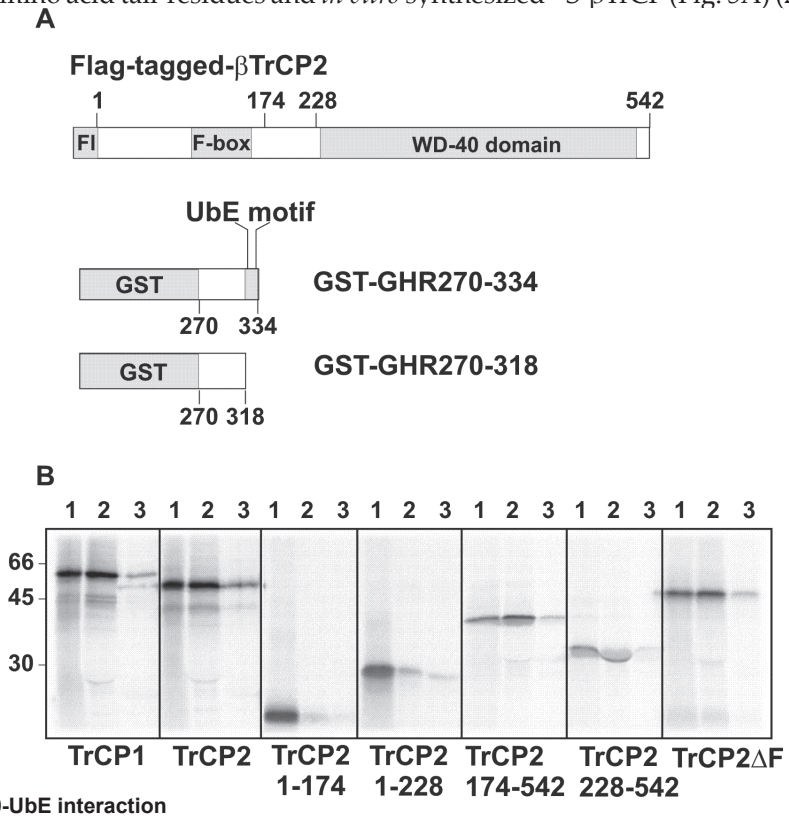

Fig. 5. WD40-UbE interaction

A. Structure of the constructs. Human TrCP2 contains 542 amino acids, including the F-box, in the N-terminal part and the 7 blades of the propeller constituting the WD40 domain. The GST-GHR fusion proteins contain the 64 and 48 membrane-proximal amino acids of the GHR cytosolic tail (GST-GHR270-334) and (GST-GHR270-318), respectively. B, 35S-in vitro labelled full-length and truncated TrCP molecules were incubated with the two GST-GHR fusion proteins for 60 minutes on ice, the beads were washed and the radioactivity was visualized using a Molecular Dynamics Phosphorimager. Lanes 1 contain16% of the input radioactivity; in lanes 2 the specific interactions with GST-GHR270-334 are visualized and in lanes 3 the binding to the UbE-deficient GST-GHR270 318. Data are representative of 2 experiments.

left panels, lanes 2, shows that both βTrCP1 and βTrCP2 bind with high efficiency to the GST-GHR270-334. If a slightly shorter fusion protein was used (270-318) the interaction was virtually abolished (lanes 3). This demonstrates an efficient interaction between βTrCP and the UbE motif in the GHR. As the binding is highly efficient at 0°C, the interaction is very likely direct and does not require any other post-translational modification. The next question is whether GHR binds to βTrCP via its WD40 domain, as has been established for other E3 substrates. Four different constructs were made, expressing βTrCP2 segments 1-174, 1-228, 174-542, and 228-542 (Fig. 5A). Using the GST-GHR fusion proteins, in vitro binding assays clearly show that the interaction is via the WD40 domain; the F-box is not necessary for interaction (right panel). In addition, specific binding was only observed if the UbE motif was present (compare lanes 2 and 3).
Together, the experiments show that βTrCP interacts with the GHR and that the F-box protein is required for GHR endocytosis. From comparison between the mutational analyses previously measured for GHR endocytosis and the results obtained with the same constructs for GHR-βTrCP interaction, we conclude that βTrCP is a prime regulator of GHR endocytosis via the UbE motif.

βTrCP isoforms

Mainly due to lack of specific antibodies, until now the role of βTrCP has not been elucidated at the level of isoform specificity. The main differences between the isoforms are located at the N-terminus. βTrCP1, in general acting on substrates involved in cell cycle regulation, is mainly present in the nucleus, while βTrCP2, presumably acting on membrane-bound substrates, resides in the cytosol (9,29). Our experiments show that both isoforms can be detected in pull-down experiments from bacterially-produced GST-GHR fusion proteins (Fig. 5B). However, only functional experiments can validate the interaction. Therefore, we determined the effect of isoform-specific βTrCP silencing on GH internalisation. We used several (combinations of) silencing oligonucleotides to specifically deplete transiently expressed flag-tagged βTrCP isoforms. Fig. 6A, lower panel shows that the siRNA(TrCP) (combi) probe silenced both isoforms, while siRNA(TrCP-1) only silenced TrCP1, and not TrCP2. SiRNA(TrCP2) silenced only TrCP2 and not TrCP1, while the combination of both TrCPs (TrCP1+2) silenced both isoforms. From the upper panel of Fig. 6A we conclude that silencing of βTrCP1 alone does not affect GH internalisation, whereas in every combination in which βTrCP2 was silenced, a clear effect on GH uptake was visible, comparable to silencing of clathrin heavy chain.
In Fig 6B we asked whether replenishment of either of the isoforms is capable of restoring the silencing phenotype. The lower panel shows that silencing is specific: the combi siRNA probe silences both isoforms (lanes 3), except if a βTrCP siRNA-resistant replacement vector was used (lanes 4). If βTrCP1- or -2-specific silencing probes were used, as expected, the 'resistant' βTrCP DNA could be silenced (lanes 5-8). Using this strategy the GH-internalisation assay shows that (over)expression of both resistant TrCP isoforms can rescue the silencing effect, indicating that both βTrCP isoforms are capable of GHR cargo selection at the cell surface. Our conclusion is that, although both βTrCP isoforms are capable of acting in GHR endocytosis, βTrCP2 is the most obvious candidate for this role in endogenous situations.

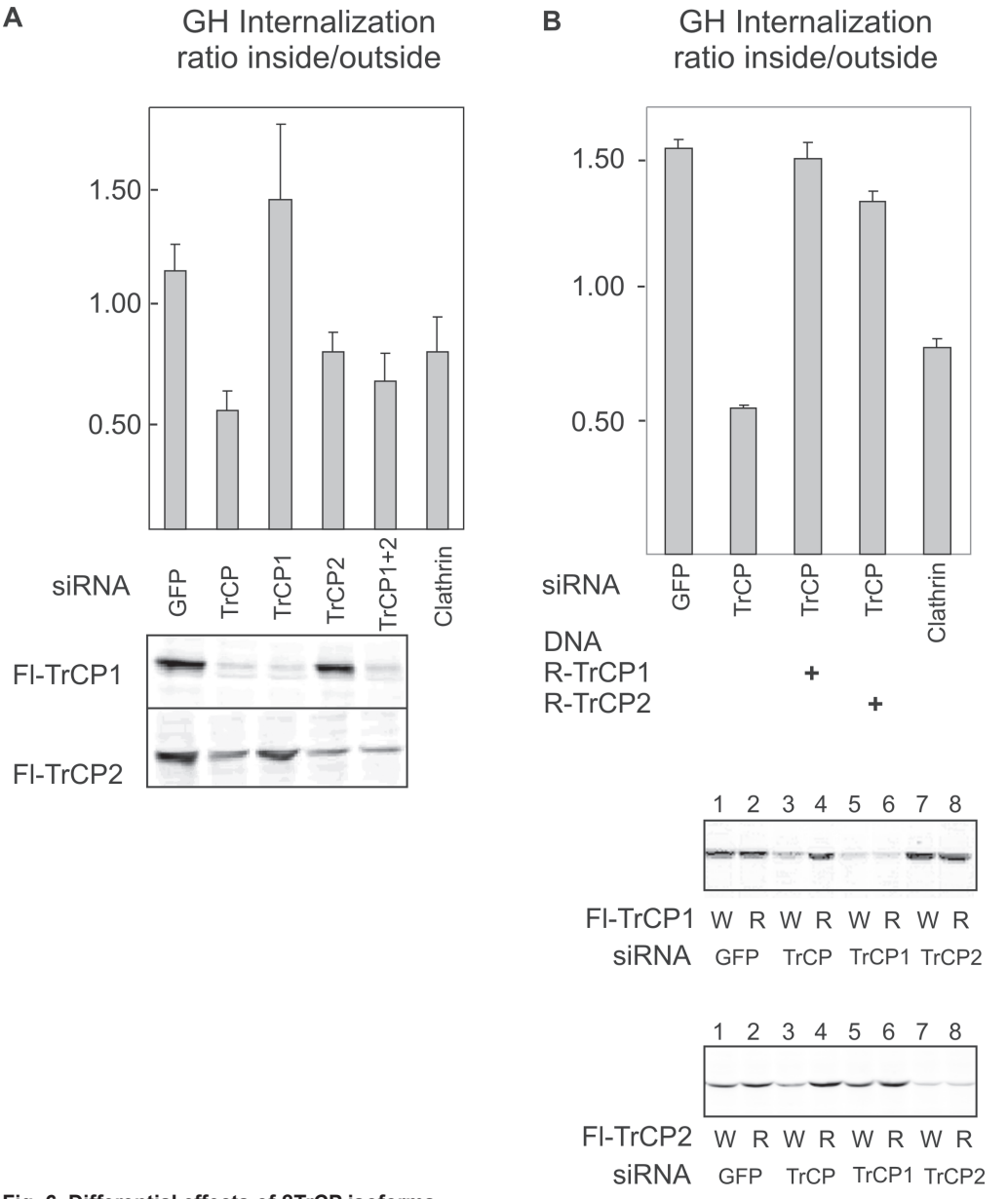

Fig. 6. Differential effects of βTrCP isoforms.
A. GHR-expressing Hek293 cells were transfected with siRNA as indicated to silence the isoforms of βTrCP or clathrin heavy chain (CHC); siRNA for GFP was applied as a control. Cells were incubated with 125I-GH for 15 min at 37°C and GH internalisation was expressed as the ratio between intracellular and extracellular radioactivity. In the lower panel, cells were transfected with either flag-tagged TrCP1 or -2 (Fl-TrCP), together with the siRNAs. As negative control GFP siRNAs were applied. SiRNAs designated TrCP represents the combi probe, silencing both isoforms. Data are representative of 4 experiments. B. GHR-expressing Hek293 cells were transfected with siRNA-resistant TrCP constructs, designated R-TrCP, to rescue the silencing. Cells were incubated with 125I-GH for 15 min at 37°C and the ratios inside/out were calculated as in A. To compare the effect of the TrCP silencing, clathrin silencing was applied. In the lower panel, the action of the different silencing experiments were controlled with vectors expressing flag-tagged TrCP (W) or siRNA-resistant TrCP (R) containing 3 silent mutations that prevents silencing by the combi probe (TrCP), but not by the specific TrCP1 or TrCP2 oligonucleotides. The blots were immunostained with anti-flag antibodies. Data are representative of 2 experiments.

Discussion

Previously, we have shown that GHR endocytosis and subsequent degradation in the lysosomes depends on the activity of the ubiquitin system via the UbE motif. Here, we identify βTrCP as a key factor required for GHR endocytosis. βTrCP specifically binds to the GHR motif that is required for ubiquitination-dependent endocytosis. These data support the conclusion that this factor recruits the ubiquitination machinery. The results with Skp1 and Neddylation strengthen our hypothesis that the complete SCF(βTrCP) complex including Skp1, Cul1, Rbx1, and an ubiquitin conjugase is present at the GHR tail. This is the first observation that shows direct involvement of a SCF E3 in endocytosis of cytokine receptors, without targeting the receptor itself for ubiquitination. Although the test system uses GHR (over)expressing cells, the experiments show that endogenous TrCP levels are sufficient to rapidly initiate GHR endocytosis and degradation. The physiological relevance of the findings is further supported by the TrCP silencing experiments: if the cellular levels of both TrCP species were diminished, uptake and degradation of GHR were inhibited.

Detailed structural information is available on the interaction between the WD40 domain of the SCF(βTrCP) with βcatenin (30). The F box protein βTrCP recognizes the dual phosphorylated DpSGFXpS destruction motif, present in βcatenin, and directs the SCF(βTrCP) E3 to ubiquitinate it at a lysine residue, 13 amino acids upstream of the destruction motif (commonly present 9-14 positions upstream). The β-catenin peptide binds the top face of the β-propeller, with the six residue destruction motif dipping into the central channel. All seven WD40 repeats contact βcatenin. Comparison with the UbE motif reveals little resemblance to the catenin destruction motif: the UbE motif is 11 amino acids long, it contains 3 acidic residues critical for binding (D321, E326, D331) instead of the two phosphorylated serine residues in βcatenin, and lacks the canonical glycine residue, present in all phosphorylated DSGFXS destruction motifs described until now (31,32). The results of Fig. 4 are most predictive and point to a binding site with EFIxxD as the strongest interacting residues. Another deviation from the canonical motif is the lack of a lysine residue at 9-14 positions upstream, although there is a conserved lysine at 315 immediately after a tyrosine that is phosphorylated upon GH binding. However, mutation of this lysine residue into arginine does not affect its ubiquitination-dependent endocytosis (22). Together, these results are in line with our previous observation that ubiquitination of the GHR is not required for endocytosis. Thus, binding of a presumed SCF(βTrCP) E3 does not target the GHR for ubiquitination but must be directed towards another factor in the GHR-ubiquitination complex (22). Before 3-D will reveal the precise interaction between the βTrCP-WD40 domain and the UbE motif, it is already clear that the UbE-WD40 interaction considerably differs from the WD40-DSGFXS destruction motif interactions described until now.

Interaction of βTrCP is the first step towards factual endocytosis of the GHR via a clathrin-mediated selection process. To involve the ubiquitination machinery in this process the GHR must be dimerized (33). Recent observations on βTrCP show that it indeed contains a dimerization domain that is important for its E3 function (34). Also cullins seem to occur as dimers in the context of SCF complexes (35). Our in vitro binding experiments with GST-GHR tails suggest that also monomeric GHR can bind βTrCP, although owing to the tendency of GST to homodimerize, it might well be that also the

GST-GHR molecules represent dimeric GHR segments (36). Previously, we reported that a chimeric GHR consisting of the extracellular domain of LDL receptor-like protein and the trans-membrane and cytosolic domain of the GHR could not dimerize anymore, and that monomeric GHR can endocytose in an ubiquitin-system independent fashion. As dimerization occurs in the ER and βTrCP is a homodimer, it is possible that βTrCP acts as a chaperone at the ER. So, it remains to be investigated whether the dimerization of βTrCP poses the necessity for dimerization on the GHR.Cargo selection via βTrCP thus requires an active E3, followed by proteasomal activity, and interaction with the clathrin-mediated machinery (22). Whether the SCF E3 ubiquitinates a factor X and enables GHR to connect to the clathrin heavy chain remains to be determined. Previously, we have identified several factors that bound specifically to the UbE motif (21). The tetratricopeptide repeat-containing protein SGT might be a factor bridging the gap between βTrCP and clathrin via the U-box ligase carboxyl-terminus of Hsc70-interacting protein (CHIP) (21,37). Another scenario might be that the ubiquitin-moiety present in the SCF(βTrCP) complex serves as a ligand for ubiquitin-binding proteins that are part of the clathrin-mediated endocytosis machinery (38).

βTrCP is involved in the degradation of prolactin and interferon receptors (17-19). ***Fuchs** et al.* identified βTrCP interaction with these receptors via their DSGFXS destruction motifs and showed that the E3 ubiquitinates both the interferon-γ and prolactin receptors, dependent on an intact DSGFXS motif (31). Upon ligand binding this motif is phosphorylated after which the SCF E3 ubiquitinates certain lysines in the tail of the receptors. This leads to their destruction. It is clear that this scenario differs from the action of βTrCP in GHR endocytosis. Although the GHR contains a DSGΦXS-like destruction motif (DSGRTS), mutation of the serine residues did not result in a shorter half-life or in inhibition of GHR endocytosis (unpublished), nor did mutation/removal of the motif interfere with the βTrCP-GHR interaction.

Ubiquitination by the SCF(βTrCP) E3 of a factor generally results in its destruction by the proteasome. In most cases the decision to degrade the target is not in the SCF(βTrCP) E3 action, but depends on the activity of one or two kinases that phosphorylate serine residues in the degradation motif. Our experiments with GST-GHR fusion proteins indicate that binding of βTrCP to the UbE motif does not depend on a preceding action of another post-translation modification. Thus, very likely, the cytosolic concentration of βTrCP determines its activity level to induce GHR endocytosis. This is reflected in several experiments showing that decreased amounts of βTrCP inhibit GH uptake and GHR degradation. Thus, the free cytosolic concentration of βTrCP may function as a factor in GH-sensitivity of cells: at low concentrations the cells are more sensitive to GH than at high concentrations. Currently, βTrCP appears to be involved in the degradation of at least 10 substrates at the same time: βcatenin, NFκB, IκB (31), Emi1 (39), Wee1 (40), ATF4 (41), hDlg (42), CDC25B (32), IFN-R1 (17), Gli3 (43), Prl-R (21). In all except CDC25B, degradation is regulated by phosphorylation. Although the two βTrCP sub-species may be differently localised in the cell they can take over their tasks if one of the two is silenced by RNA interference techniques. As probably no modification is required for βTrCP to bind GHR, the two proteins might regulate each other's activity: more cellular βTrCP results in increased GHR endocytosis/degradation, while more GHR might absorb free βTrCP, immediately affecting the other βTrCP functions. Evidence for this comes also from the observation that hnRNP-U acts as a pseudosubstrate (44). Generally, βTrCP is part of an E3 complex involved in degradation of key factors

controlling cell growth, survival and transformation (31). As it is a short-lived protein itself, more studies are needed to understand the consequences of βTrCP concentrations for cellular regulation, and the GHR activity in particular. The GH-IGF1 system is involved in longevity, cell cycle, apoptosis and immunity (1): in catabolic (cachectic) conditions, as old age and cancer, unknown stress signals turn the system off resulting in GH-insensitivity, while anabolic conditions require cells to be highly GH-responsive. Whether and how βTrCP is at this balance remains to be elucidated.

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References

1. Sinclair, D. A. (2005) *Mech Ageing Dev* **126**, 987-1002
2. Waters, M. (1999) *The growth hormone receptor*, Oxford University Press, New York
3. Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., and Wood, W. I. (1987) *Nature* **330**, 537-543
4. Argetsinger, L. S., Campbell, G. S., Yang, X. N., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N., and Carter Su, C. (1993) *Cell* **74**, 237-244
5. Alves dos Santos, C. M., ten Broeke, T., and Strous, G. J. (2001) *J Biol Chem* **276**, 32635-32641.
6. Strous, G. J., dos Santos, C. A., Gent, J., Govers, R., Sachse, M., Schantl, J., and van Kerkhof, P. (2004) *Curr Top Microbiol Immunol* **286**, 81-118
7. Hershko, A., Ciechanover, A., and Varshavsky, A. (2000) *Nat Med* **6**, 1073-1081
8. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev.* **13**, 270-283
9. Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Suzuki, T., Ichiyama, A., Ikenoue, T., Omata, M., Furuichi, K., and Tanaka, K. (1999) *Biochem. Biophysic. Res. Commun.* **256**, 127-132
10. Traub, L. M., and Lukacs, G. L. (2007) *J Cell Sci* **120**, 543-553
11. Galan, J. M., and Haguenauer-Tsapis, R. (1997) *EMBO Journal* **16**, 5847-5854
12. Hicke, L., and Dunn, R. (2003) *Annu Rev Cell Dev Biol* **19**, 141-172
13. Roth, A. F., and Davis, N. G. (2000) *J. Biol. Chem.* **275**, 8143-8153
14. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) *Science* **294**, 1307-1313
15. Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) *EMBO J.* **16**, 6325-6336
16. Strous, G., and Gent, J. (2002) *FEBS Lett* **529**, 102-109
17. Kumar, K. G., Tang, W., Ravindranath, A. K., Clark, W. A., Croze, E., and Fuchs, S. Y. (2003) *EMBO J.* **22**, 5480-5490
18. Kumar, K. G. S., Krolewski, J. J., and Fuchs, S. Y. (2004) *J. Biol. Chem.* **279**, 46614-46620
19. Li, Y., Suresh Kumar, K. G., Tang, W., Spiegelman, V. S., and Fuchs, S. Y. (2004) *Mol. Cell. Biol.* **24**, 4038-4048
20. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) *EMBO J.* **15**, 3806-3812
21. Schantl, J. A., Roza, M., de Jong, A. P., and Strous, G. J. (2003) *Biochem. J.* **373**, 855-863
22. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) *EMBO J.* **18**, 28-36.
23. van Kerkhof, P., Govers, R., Alves dosSantos, C. M. A., and Strous, G. J. (2000) *J Biol Chem* **275**, 1575-1580
24. van Kerkhof, P., Sachse, M., Klumperman, J., and Strous, G. J. (2000) *J. Biol. Chem.* **276**, 3778-3784.
25. Ohh, M., Kim, W. Y., Moslehi, J. J., Chen, Y., Chau, V., Read, M. A., and Kaelin, W. G., Jr. (2002) *EMBO*

Rep **3**, 177-182
26. Sadot, E., Simcha, I., Iwai, K., Ciechanover, A., Geiger, B., and Ben-Ze'ev, A. (2000) *Oncogene* **19**, 1992-2001
27. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) *EMBO J.* **16**, 4851-4858
28. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinology* **143**, 1243-1252
29. Besnard-Guerin, C., Belaidouni, N., Lassot, I., Segeral, E., Jobart, A., Marchal, C., and Benarous, R. (2004) *J Biol Chem* **279**, 788–795
30. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) *Mol Cell* **11**, 1445-1456
31. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23**, 2028-2036
32. Kanemori, Y., Uto, K., and Sagata, N. (2005) *Proc Nat Acad Sci USA* **102**, 6279-6284
33. Gent, J., van Kerkhof, P., Roza, M., Bu, G., and Strous, G. J. (2002) *Proc Nat Acad Sci USA* **99**, 9858-9863
34. Suzuki, H., Chiba, T., Suzuki, T., Fujita, T., Ikenoue, T., Omata, M., Furuichi, K., Shikama, H., and Tanaka, K. (2000) *J. Biol. Chem.* **275**, 2877-2884
35. Chew, E. H., Poobalasingam, T., Hawkey, C. J., and Hagen, T. (2006) *Cell Signal,* Epub ahead of print.
36. Shih, S. C., Katzmann, D. J., Schnell, J. D., Sutanto, M., Emr, S. D., and Hicke, L. (2002) *Nat Cell Biol* **4**, 389-393
37. Timsit, Y. E., Miller, S. L., Mohney, R. P., and O'Bryan, J. P. (2005) *Biochem. Biophys. Res. Commun.* **328**, 550-559
38. d'Azzo, A., Bongiovanni, A., and Nastasi, T. (2005) *Traffic* **6**, 429-441
39. Peters, J. M. (2003) *Mol Cell* **11**, 1420-1421
40. Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Watanabe, N., Hunter, T., and Osada, H. (2004) *Proc Nat Acad Sci USA* **101**, 4419-4424
41. Lassot, I., Segeral, E., Berlioz-Torrent, C., Durand, H., Groussin, L., Hai, T., Benarous, R., and Margottin-Goguet, F. (2001) *Mol Cell Biol* **21**, 2192-2202.
42. Mantovani, F., and Banks, L. (2003) *J. Biol. Chem.* **278**, 42477-42486
43. Wang, B., and Li, Y. (2006) *Proc Nat Acad Sci USA* **103**, 33-38
44. Davis, M., Hatzubai, A., Andersen, J. S., Ben-Shushan, E., Fisher, G. Z., Yaron, A., Bauskin, A., Mercurio, F., Mann, M., and Ben-Neriah, Y. (2002) *Genes Dev.* **16**, 439-451

CHAPTER III

Jak2 is a negative regulator of ubiquitin-dependent endocytosis of the growth hormone receptor

Joyce Putters, Peter van Kerkhof, Agnes G.S.H. van Rossum, Ana Gracanin and Ger J. Strous

Department of Cell Biology and Institute of Biomembranes
University Medical Center Utrecht

Manuscript submitted

Abstract

Length and intensity of signal transduction via cytokine receptors is precisely regulated. Degradation of certain cytokine receptors is mediated by the ubiquitin ligase SCF(βTrCP). In several instances, Janus kinase (Jak) family members can stabilise their cognate cytokine receptors at the cell surface. In this study we show that Jak2 binding to the growth hormone receptor (GHR) prevents endocytosis in a non-catalytic manner. Following receptor activation, the detachment of phosphorylated Jak2 induces down regulation of the GHR by SCF(βTrCP). We show that both GH-induced and constitutive GHR endocytosis depend on the same factors, strongly suggesting that the modes of endocytosis are identical. Both Jak2 and βTrCP bind to neighbouring linear motifs in the GHR tail without the requirement of modifications, suggesting that GH sensitivity is regulated by the cellular level of non-committed Jak2. As many cytokine receptors depend on Jak2, the study suggests an integrative role of Jak2 in cytokine responses.

Introduction

In mammalian cells the Janus kinase (Jak) family of protein tyrosine kinases comprises Jaks1-3 and Tyk2. All Jak family members are widely expressed except for Jak3 that is restricted to cells of the hematopoietic system. Jaks associate non-covalently with a membrane-proximal region in cytoplasmic tails of cytokine receptors and play crucial roles in the initial steps of cytokine signaling (10, 16).

At the C-terminus, Jak family members contain the tyrosine kinase domain preceded by a pseudokinase domain. The N-terminal half of Jak comprises a postulated FERM domain (four-point-one, ezrin, radixin, moesin) that binds to cytokine receptors and a potential SH2 domain (11). Specific mutations in the FERM domain inhibit cytokine receptor association and concomitantly abrogate Jaks' ability to respond to ligand binding. Binding of the FERM domain to receptors has also been proposed to assist in cell surface localisation of several cytokine receptors (8, 13-15, 25, 26). Growth hormone (GH) is a multifunctional, clinically important cytokine hormone that acts through its type I cytokine receptor, the growth hormone receptor (GHR). The type I cytokine receptor family further includes the prolactin (Prl) receptor, the erythropoietin (Epo) receptor, and the thrombopoietin (Tpo) receptor (4). All cytokine receptors lack intrinsic kinase activity. Instead, a conserved proline-rich domain in their cytosolic tail, box-1, functions as a binding site for Jak family members. In the case of GHR, ligand binding results in the activation of Jak2 molecules (2) that in turn phosphorylate tyrosine residues in itself, the receptor's cytosolic tail and downstream signalling molecules (18). Both liganded and unoccupied GHRs are endocytosed via clathrin-coated vesicles and subsequently transported via endosomes to lysosomes (29). Previously, we have shown that both endocytosis and transport to lysosomes require an active ubiquitin conjugation system and a 10-amino acid motif (UbE-motif inside the conserved box-2 region) in the cytosolic tail of GHR (33). The SCF(βTrCP) ligase drives endocytosis and degradation of the GHR by binding directly and specifically with its WD40 domain to the non-conventional UbE motif of GHR (35).

More than 400 actions are mediated by GH via the GHR and they depend on relative rotation of the pre-dimerised receptor subunits aligning two Jak2 molecules (9, 36). Recent studies show that the Jak family also plays non-catalytic roles in regulating the cellular localization and traffic of cytokine receptors (25, 28). For the GHR, increased Jak2 expression levels increase the fraction of mature GHRs (13).

GHR endocytosis provides an important means to control GH sensitivity. Patients with cancer-induced wasting are relatively GH-insensitive and have low GH binding protein titers, indicative for rapid degradation of GHRs (1). It is therefore important to understand the cellular mechanisms involved in the regulation of GHR endocytosis.

Here, we demonstrate that Jak2 binding specifically inhibits GHR endocytosis, independent of its kinase activity. A study of Funakoshi-Tago *et al* (7) showed that phosphorylation of tyrosine 119 of Jak2 is sufficient to abrogate the interaction of Jak2 with several cytokine receptors. Based on this, we hypothesized that GH stimulation releases Jak2 from the GHR. Our experiments show that Jak2 activation indeed results in release of phosphorylated Jak2 from the GHR, after which the GHR is allowed to internalise via SCF(βTrCP)-mediated endocytosis and is degraded. In addition, GH-induced and constitutive GHR endocytosis require the same factors, strongly suggesting that the pathways are identical.

Experimental procedures

Materials, antibodies and DNA constructs

MβCD and staurosporin were purchased from Sigma. MG132 was obtained from Calbiochem. GHR antisera were described before (29). Monoclonal 4G10 anti-pY was obtained from Upstate (Millipore). Monoclonal antibodies against Jak2 (AHO1352), ubiquitin (clone FK2) and clathrin (C1860) were bought from Biosource, Biomol and Sigma, respectively. Anti-STAT5 (C17) was obtained from Santa Cruz Biotechnologies Inc. and anti-actin (Clone C4) was obtained from MP Biomedicals Inc. Monoclonal anti-Flag (M2) was from Sigma. Polyclonal anti-Jak2 was described previously (30) and anti-βTrCP1 was described before (35). Full-length rabbit GHR cDNA in pcDNA3 has been described before (29), just as GHR(S366A, S370A) (DSG mutant) (35). The Flag-tagged wild type mouse Jak2 constructs were a generous gift from Prof. Carter-Su. Flag-Jak2(1-525) was constructed by the introduction of a stop-codon using the Quickchange mutagenesis kit from Stratagene. Jak2(Y119E), the GHR in which all tyrosines were mutated to phenylalanines and the GHR in which 326EFIxxD residues were mutated to alanine, were also produced using the same kit. Protein A-beads were from Repligen. We cloned Jak2 into vector pSG213 for stable expression in γ2A cells. pSG213 was a kind gift of Frauke Melchior.

Cell culture, transfections, and microscopy

HEK293 cells, stably expressing wtGHR were maintained in DMEM high glucose (4.5 g/l), supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 600 μg/ml G418. IM9 cells were maintained in RPMI 1640, 10% FCS, penicillin, streptomycin, containing 4.5 g/l glucose and 1 mM sodium pyruvate. γ2A cells were maintained in DMEM low glucose (1.0 g/l), supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. Stable cell lines with Jak2 in pSG213 were maintained in maintenance medium, supplemented with puromycin and stable cell lines with wtGHR with hygromycin. When indicated, the cells were pretreated with 10 mM MβCD, 20 μM MG132 or staurosporin in serum free medium for 60 minutes at 37°C. Human GH was added at a concentration of 180 ng/ml. DNA transfections were performed using FuGene 6 (Roche, Applied Sciences) according to the manufacturer's instructions. Clathrin was silenced using the ON-TARGETplus SMARTpool of Dharmacon (Thermo Fisher Scientific., Lafayette, CO). βTrCP (1 and 2) was silenced with the "combi probe" and GFP siRNA was used as described before (35). In all siRNA transfections Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions. Microscopy studies were performed as previously described (35).

Lysis and immunoprecipitations

For GHR-Jak2 co-immunoprecipitations, the cells were lysed in 20 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin. For phosphorylation and ubiquitination experiments, the cells were lysed in 1% Triton X-100 with inhibitors (1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM NEM, 1 mM Na_3VO_4 and 50 mM NaF). Cell lysates were centrifuged to pellet the nuclei and the supernatants were used for GHR isolation in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% BSA, and inhibitors via immune precipitation with

anti-GHR and protein A beads. Immunoprecipitates were subjected to reducing SDS-PAGE and transferred to Immobilon-FL polyvinylidenedifluoride membrane (Millipore). Blots were immunostained with the indicated primary antibodies followed by Alexa Fluor 680, Alexa-800 IRDye conjugated anti-mouse or anti-rabbit antibodies. Detection was performed with an Odyssey system (LI-COR Biosciences).

^{125}I-GH binding and internalisation and cell fractionation

^{125}I-GH binding and internalization was performed as previously described (33). Internalisation was expressed as a ratio between iodinated GH inside and total. For cell fractionation, 10^7 (IM9) cells were incubated for 10 minutes with GH and washed with cold PBS. Cell fractionation was performed as previously described (3), except that we omitted the 12.500g centrifugation step. After fractionation, the membrane and cytoplasmic fractions were immunoprecipitated with polyclonal anti-Jak2, followed by isolation using protein A beads. The samples were subjected to gel electrophoresis and western blotting as described above.

RNA isolation and Real time quantitative–PCR (qRT-PCR)

Total RNA from IM9, HEK293 and HepG2 cells was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Synthesis of cDNA was carried out from 1μg total RNA in 20 μl reaction volumes using the iScript™ cDNA synthesis kit according to the manufacturer's protocol (BioRad). Primers were designed using primer select software of DNA star (Madison, WI) according to the parameters outlined in the BioRad i-cycler manual. The specificity of each primer was confirmed by sequencing its product. Rps19 and β-actin genes were used as the non-regulated reference genes for normalization of target gene expression. Sequences of the used primers are for Jak2 TGAAGACCGGGATCCTACACACAGTT (forward) and GTCATACCGGCACATC-TCCACAC (reverse), for βTrCP2 CATGTTGCAGCGGGACTTTATTACC (forward) and GATCACTCGCTGCCATTCTTTACAT (reverse), for Rps19 CCTTCCTCAAAAAGTCTGGG (forward) and GTTCTCATCGTAGGGAGCAAG (reverse), for β-actin. TCCCTGGAGAAGAGCTACG (forward) and GTAGTTTCGTGGATGCCACA (reverse). Annealing temperatures for Jak2, βTrCP2, Rsp19 and β-actin were 63.0, 62.0, 61.0 and 60.0 respectively. QRT-PCR was performed using BioRad MyIQ detection system (BioRad) with SYBR green fluorophore. Data analysis was carried out using the pair wise fixed reallocation and randomization test incorporated in the software program REST-MCS (23) at 5% level of significance. Experiments were performed in duplicate and values from six experiments were used for data analysis.

Results

Jak2 specifically inhibits GHR endocytosis independent of its kinase activity

Recently, we showed that the E3 ligase SCF(βTrCP) is required for GHR endocytosis via the UbE-motif of the GHR (35). Other studies revealed that Jak2 stabilises the GHR (5, 13). To investigate whether this is due to Jak2-mediated inhibition of GHR endocytosis, we expressed Jak2 in GHR-expressing cells and incubated the cells with Cy3-GH to monitor GHR endocytosis. Cells overexpressing Jak2 showed a strong increase of Cy3-GH labelling at the cell surface compared to control cells (EV) (Fig. 1A).

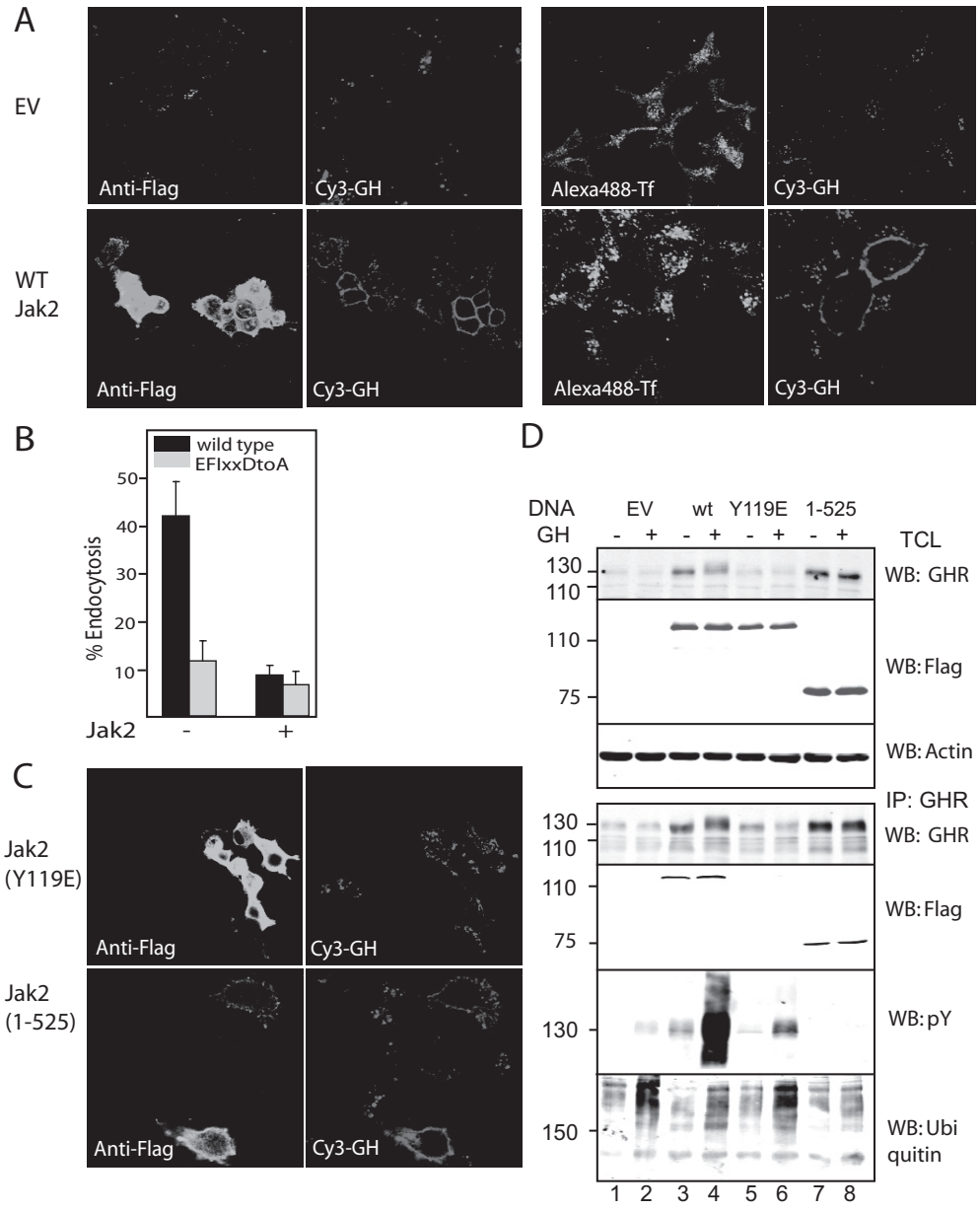

Fig. 1. Jak2 inhibits GHR endocytosis. A. GHR-expressing HEK293 cells were transfected with empty vector
(EV) or Flag-Jak2. One coverslip was incubated with Cy3-GH, fixed and stained with anti-Flag (left panel). For EV and Jak2-transfected cells, cells were incubated with Cy3-GH and Alexa488-transferrin (right panel). B. GHR or GHR(EFIxxDtoA)-expressing cells were incubated for 2 h on ice with 180 ng/ml 125I-GH. Unbound label was removed and the cells were incubated at 37°C for 10 min. The percentage of endocytosis was calculated; the diagram represents the mean values of two experiments ± SD. C. GHR-expressing HEK293 cells were transfected with Jak2(Y119E) or Jak2(1-525) and the cells were incubated with Cy3-GH, fixed and stained with anti-Flag. D. Cells were transfected with empty vector (EV), wild type Jak2, Jak2(Y119E) or Jak2(1-525) and treated with GH for 15 minutes as indicated. Total cell lysates (TCL, upper panel) and GHR immuno¬precipitations (lower panel) were analysed on western blot (WB) using the indicated antibodies. 130, mature GHR; 110, precursor GHR. Data are representative of three experiments.

Importantly, transferrin uptake, via the constitutively recycling transferrin receptor, was unaffected. To confirm that Jak2 indeed inhibits GHR endocytosis we used a ^{125}I-GH uptake assay. As seen in Fig. 1B, about 40% of ^{125}I-GH was endocytosed within 10 minutes, whereas less than 10% endocytosis occurred if Jak2 was co-expressed, confirming that Jak2 acts as an inhibitor of GHR endocytosis. Conversely, when cells expressing a βTrCP binding mutant of GHR were used, only 15% endocytosis occurred. Co-expression of Jak2 had no significant additive effect on GHR endocytosis. Together, these results demonstrate that overexpression of Jak2 inhibits GHR endocytosis.

To demonstrate that interaction between GHR and Jak2 is required for this inhibition, we used a Jak2-binding mutant. Previously, Funakoshi-Tago and co-workers showed that mimicking a phosphorylated tyrosine residue by glutamic acid at position 119 abrogates Jak2 binding to a subset of cytokine receptors, including the GHR (7). Fig. 1C clearly shows that expression of Jak2(Y119E) did not affect Cy3-GH uptake. In agreement with the results of Funakoshi-Tago *et al*, Jak2(Y119E) did not co-precipitate with the GHR (Fig. 1D). These data show that Jak2 binding to the GHR is required for inhibition of GHR endocytosis.

In order to find out whether kinase activity to inhibit GHR endocytosis, we expressed a truncation mutant Jak2(1-525), in which the pseudokinase and the kinase domain were deleted. Jak2(1-525) inhibited GHR endocytosis similar to wild type Jak2, as shown by a Cy3-GH uptake assay (Fig. 1C). Fig. 1D shows that Jak2(1-525) was not able to phosphorylate the GHR, confirming the absence of kinase activity. Thus, Jak2 kinase activity is not necessary for inhibition of GHR endocytosis.

Upon assessment of GHR stabilization, we found that both wild type Jak2 and Jak2(1-525) strongly stabilised the GHR, whereas Jak2(Y119E) did not (Fig. 1D, upper panel, lanes 1, 3, 5 and 7). These data are in agreement with the Cy3-GH and ^{125}I-GH uptake assays (Fig. 1A-C). Although Jak2(Y119E) and wild type Jak2 expression levels were comparable, Jak2(Y119E) expressing cells had a much lower level of GH-induced GHR phosphorylation, indicating that Jak2-GHR binding is essential for GHR phosphorylation (Fig. 1D). Together, we conclude that Jak2 binding to the GHR inhibits GHR endocytosis independent of Jak2 kinase activity.

Jak2 acts upstream of SCF(βTrCP)

Based on our conclusion that Jak2 binding is sufficient to inhibit GHR endocytosis, we hypothesized that Jak2 keeps the GHR at the cell surface by preventing it from entering into the process of endocytosis (cargo selection). As we have previously shown, cargo selection is performed by the ubiquitin ligase SCF(βTrCP) (35). Although GHR does not seem to be an essential target for ubiquitination, the receptor becomes ubiquitinated during the process of endocytosis (34). As the genuine substrate for SCF(βTrCP) is unknown, we used GHR ubiquitination to measure whether GHR had entered the cargo selection process. If SCF(βTrCP) is indeed involved in cargo selection, βTrCP gene silencing must prevent both GHR ubiquitination and degradation. Fig. 2A clearly shows accumulation of the mature GHR, whereas both constitutive and GH-induced GHR ubiquitination were decreased upon βTrCP silencing. In addition, we show in HEK293 cells that transfection of ubiquitin K48R completely inhibited GHR ubiquitination, whereas transfection of ubiquitin K63R did not affect GHR ubiquitination at all.

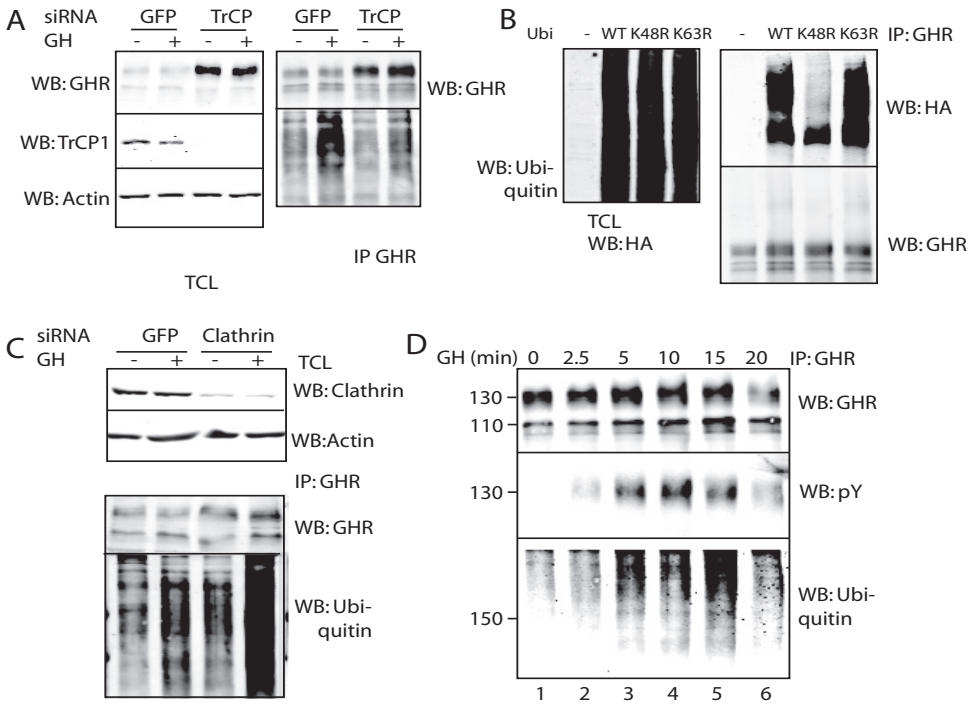

Fig. 2. SCF(βTrCP) and clathrin act downstream of Jak2.
A. Cells were either silenced for GFP as a negative control or for βTrCP and then treated for 15 minutes with GH as indicated. GHR immunoprecipitations were analysed for ubiquitina¬tion. B. Cells were transiently transfected with HA-tagged wild type ubiquitin, ubiquitin K48R or ubiquitin K63R. Total cell lysates (TCL) and GHR immunoprecipitates were analysed on western blot using the indicated antibodies. C. Cells were either silenced for GFP or for clathrin and then treated for 15 minutes with GH as indicated. Total cell lysates (TCL) and GHR immunoprecipitations were analysed with the indicated antibodies. D. GHR-expressing HEK293 cells were treated with GH as indicated. GHR immunoprecipitations were analysed on western blot with the indicated antibodies. 130,mature GHR, 110, precursor GHR. Data in A, B, C and D are representative of three experiments.

βTrCP has been shown to predominantly ubiquitinate substrates via K48 linkages (6, 12, 22, 24), so βTrCP very likely ubiquitinates GHR, although we cannot exclude the involvement of other E3 ligases. Thus, βTrCP is essential for GHR K48-linked ubiquitination. As it is obvious that clathrin-mediated endocytosis occurs *after* cargo selection by βTrCP, silencing of clathrin should accumulate ubiquitinated GHRs. This is shown in Fig. 2C, lower panel. Thus, the conclusion is justified that GHR ubiquitination occurs after cargo selection and before endocytosis and depends on SCF(βTrCP) activity. If Jak2 binding inhibits GHR endocytosis *before* cargo selection by SCF(βTrCP), GHR in complex with wild type Jak2 should accumulate at the cell surface in a non-ubiquitinated state. Therefore, we overexpressed wild type and mutant Jak2 in GHR-overexpressing cells and measured GHR ubiquitination. Upon wild type Jak2 and Jak2(1-525) overexpression, GHR was stabilised, but GHR ubiquitination was decreased, demonstrating a strong inhibition of GHR ubiquitination (Fig. 1D, lower panel, lane 4). As expected, Jak2 overexpression resulted in a strong increase in GHR phosphorylation. Since not all GHR-expressing cells were transfected with the Jak2 constructs, low levels of GHR ubiquitination resulted most

likely from non-transfected cells. As expected, the binding mutant Jak2(Y119E) showed GHR phosphorylation and ubiquitination comparable to control cells (EV).

Our data imply that Jak2 and βTrCP may act in sequence, whereby Jak2 and βTrCP activity are represented by phosphorylation and ubiquitination respectively. Therefore, the kinetics of both GH-induced ubiquitination and phosphorylation were measured within one experiment. Fig. 2D indeed shows that the maximal phosphorylation precedes ubiquitination by 5 minutes. Importantly, if GHR does not bind Jak2, the receptors are endocytosed constitutively, as demonstrated by the level of ubiquitinated GHRs that is observed without GH stimulation.

We show here for the first time that βTrCP is essential for K48-linked ubiquitination of the GHR. In addition, we conclude that Jak2 binding inhibits GHR endocytosis upstream of SCF(βTrCP) action.

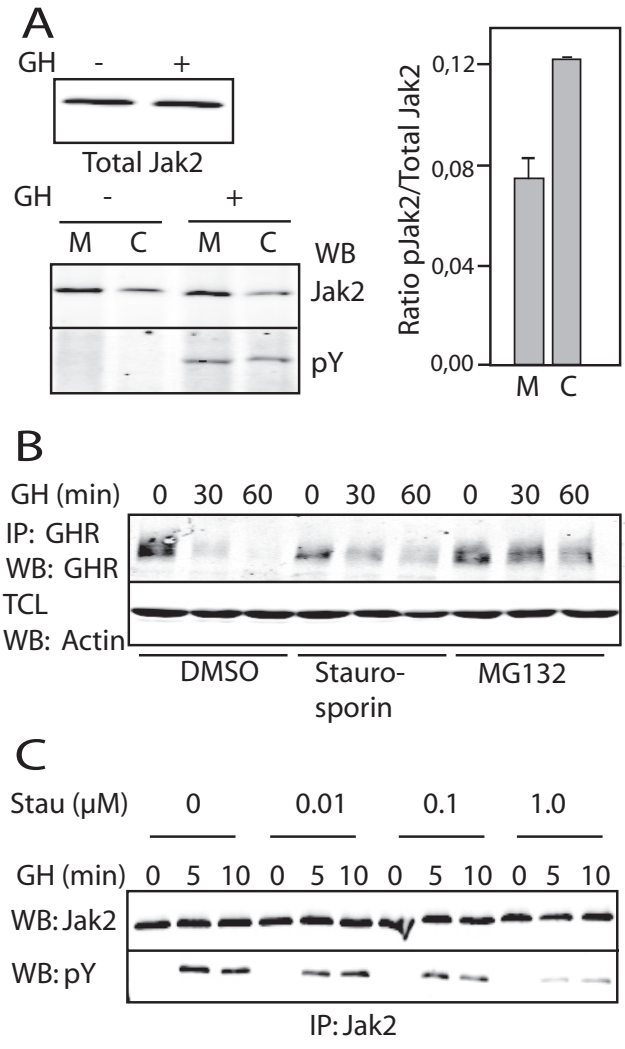

Fig. 3. GH induces Jak2 phosphorylation and release from the GHR.

A. Cells were treated with GH as indicated, followed by cell fractionation. Membrane (M) and cytosol (C) fractions were immunoprecipitated for Jak2 and analyzed with the indicated antibodies. The ratio of phosphorylated Jak2 (pJak2) and total Jak2 was calculated for each fraction. B. Cells were pretreated with dimethylsulfoxide staurosporin or MG132 for 1 hour and stimulated with GH as indicated. Total cell lysates (TCL) and GHR immunoprecipitations were analysed on western blot with the indicated antibodies. C. IM9 cells were pretreated with different concentrations of staurosporin for 1 hour followed by incubation with GH as indicated. Jak2 immunoprecipitations were analyzed with the indicated antibodies. Data in A, B, and C are representative of three experiments.

Phosphorylated Jak2 dissociates from the GHR

So far, we have shown that Jak2 inhibits GHR endocytosis non-catalytically and prevents the receptor from SCF(βTrCP)- and clathrin-mediated endocytosis. To allow GHR endocytosis, Jak2 needs to detach from the receptor. Phosphorylation of Y119 of Jak2 might provide for such a mechanism, which would imply that GH stimulation results in Jak2 release. As most studies have been performed with cell systems expressing exogenous GHR, large numbers of receptors relative to low numbers of (endogenous) Jak2 might disturb the control of endocytosis that is based on a stoichiometric relation between Jak2 and GHR.

Human IM9 lymphoblasts have detectable levels of endogenous GHR and have been used in many cytokine receptor studies. If IM9 cells contain sufficient amounts of Jak2 to accumulate endogenous GHRs at the cell surface, we expect that GH stimulation triggers GHR endocytosis by Jak2 phosphorylation (on Y119) and Jak2 detachment from the GHR. Initially, we attempted to test the endogenous Jak2-GHR interaction by co-immunoprecipitation, but were unsuccessful due to high unspecific Jak2 binding to agarose- and Ni-beads. Therefore, we based our experiments on the findings of Behrmann *et al* who showed that Jak1 and -2 mainly occur membrane-bound (3). As Jak2 can only be phosphorylated if attached to box-1 of a cytokine receptor (10, 20), this implies that phosphorylated Jak2, if present in the cytosol has been released from GHRs. To test this, IM9 cells were treated with GH and membrane and cytosol fractions were analysed for phosphorylated Jak2. Upon GH stimulation, neither total Jak2 levels nor Jak2 distribution over membrane and cytosol fractions were significantly changed (Fig. 3A). This was expected, since Jak2 is bound to a variety of other substrates at the membrane. Importantly, the fraction of phosphorylated Jak2 was considerably higher in the cytosol compared to that in the membrane fraction (Fig. 3A). These results demonstrate that phosphorylated Jak2 dissociates from the GHR upon GH stimulation. As it is unknown whether each receptor holds two Jak2 molecules at steady state, or in what priority and proportion the different tyrosine residues in Jak2 are phosphorylated, it is difficult to assess why not all phosphorylated Jak2 is in the cytosol. Most likely, Y119 is not the first tyrosine being phosphorylated after GH binding, although cytosolic phosphatases may play a role as well.

To ascertain that GH-induced GHR endocytosis in IM9 cells depends on the ubiquitin system as previously shown for exogenous GHRs in various tissue culture cells, we used the proteasomal inhibitor MG132 (34). Pretreatment of IM9 cells with MG132 indeed completely inhibited GH-induced GHR degradation (Fig. 3B, lanes 7-9). To investigate whether GH-induced endocytosis depends on Jak2 activity, we inhibited endogenous Jak2 kinase activity with the kinase inhibitor staurosporin and treated the cells with GH (Fig. 3B, lanes 4-6). Previously, it has been shown that 1 μM staurosporin almost completely abolished GHR phosphorylation in CHO cells (30). Fig. 3C shows the same result for Jak2 phosphorylation. Without staurosporin treatment, the GHR is rapidly degraded; after 60 minutes of GH stimulation hardly any GHR was detectable (Fig. 3B, lanes 1-3). In contrast, staurosporin treatment stabilised the mature GHR (Fig. 3B, lanes 4-6). In addition, we found that stimulation with GH antagonist, which is unable to induce the conformational change necessary for GHR activation upon GH stimulation, did not result in GHR degradation (unpublished data). These results strongly suggest the notion that Jak2 activity is required to induce GHR cargo selection and endocytosis by Jak2 dissociation.

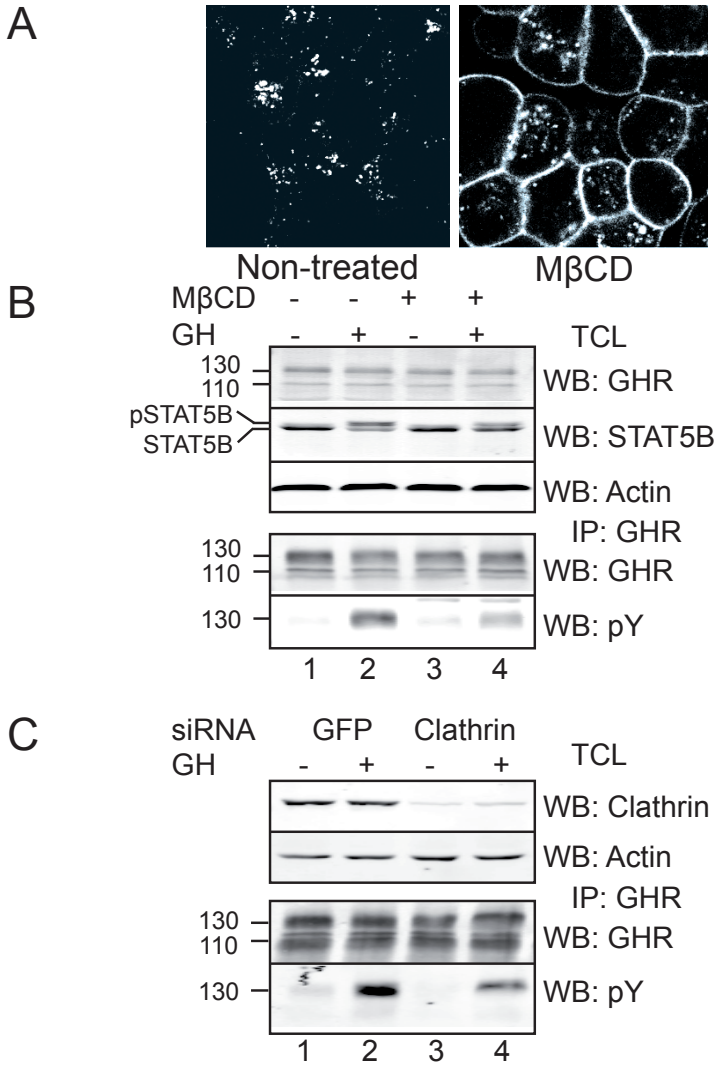

Fig. 4. Decreased Jak2 activity in coated pit.
A. GHR-expressing HEK293 cells were pretreated with MβCD for 1 hour, then incubated with Cy3-GH for 30 minutes and fluorescence was visualized with a confocal microscope. B. Cells were pretreated as in 2A, followed by incubation for 15 minutes with GH as indicated. Total cell lysates (TCL, upper panel) and GHR immunoprecipitations (lower panel) were analysed on western blot with the indicated antibodies. 130, mature GHR; 110, precursor GHR. C. Cells were silenced either for clathrin or for GFP (as a negative control), followed by incubation for 15 minutes with GH as indicated. Total cell lysates (TCL, upper panel) and GHR immunoprecipitations (IP, lower panel) were analysed on western blot with the indicated antibodies. 130, mature GHR; 110, precursor GHR. Data are representative of two experiments (B).

Decreased Jak2 activity in coated pits

As additional and independent evidence for an early role of Jak2 in the regulation of GHR endocytosis, we tested whether Jak2 activity is absent from a complex that is already selected for endocytosis, i.e. present in or at the coated pits. To probe this, we inhibited endocytosis at the coated pits by using methyl-β-cyclodextrin (MβCD). MβCD depletes cholesterol from the plasma membrane and inhibits clathrin-coated pit budding.

This causes inhibition of internalization of the transferrin receptor by 85% (31). Fig. 4A shows inhibition of GHR uptake at the cell surface after treatment with MβCD as compared to non-treated cells. Next, we stimulated with GH and measured both GHR- and STAT5 phosphorylation. Since Jak2 binds aspecifically to beads we were not able to show a direct GH-effect on the GHR-Jak2 interaction. Therefore, the levels of GHR phosphorylation were taken as an indication for Jak2 presence in the GHR complex. As seen in Fig. 4B, GHR phosphorylation was strongly inhibited in MβCD-treated cells as compared to non-treated cells that expressed comparable amounts of GHRs (lower panel, lanes 2 and 4). In addition, phosphorylation of STAT5, a major downstream signal transduction molecule, was decreased (upper panel, lanes 2 and 4). MβCD treatment did not affect the efficiency of GH-GHR interaction (Fig. 4A) indicating a strong reduction of Jak2 activity at this stage of endocytosis. This was confirmed by clathrin silencing, which resulted in a 40% reduction of GHR phosphorylation (Fig. 4C, lower panel). The reduction in GHR phosphorylation was not as prominent as for MβCD, probably due to the effectiveness of the gene silencing. These data show that GHRs that are selected for endocytosis can no longer be phosphorylated, suggesting the absence of Jak2 in the GHR complex at this stage.

GH-induced and constitutive endocytosis use the same endocytosis mechanism

To be able to compare GH-induced and constitutive GHR endocytosis we used a γ2A, human fibroblast cell line that stably expresses GHR and is Jak2 deficient (γ2A_GHR). This cell line was used to make a variant cell line that produces low numbers of Jak2 (γ2A_GHR_Jak2). Upon stimulation of both cell lines with GH, quantification of mature GHR levels showed that after 90 minutes about 50% of the GHRs were degraded in γ2A_GHR_Jak2 cells, while in γ2A_GHR cells GHR steady state levels remained unchanged (Fig. 5A). These data indicate that GH is only able to induce GHR endocytosis in the presence of Jak2. It is important to note that, without GH stimulation, the GHR steady state level in γ2A_GHR cells and γ2A_GHR_Jak2 cells is defined by constitutive GHR endocytosis. We hypothesized that GH-induced and constitutive GHR endocytosis use the same endocytosis mechanism. We reasoned that GH-induced GHR endocytosis should use the UbE motif and the SCF(βTrCP) ligase for cargo selection, should be dependent on clathrin and that tyrosine phosphorylation of the GHR tail should not affect GHR endocytosis, just as is shown for constitutive GHR endocytosis.

First, we tested whether GH-induced GHR endocytosis is ubiquitin-system dependent by treating the cells with the proteasome inhibitor MG132 and comparing MG132-treated with non-treated γ2A_GHR_Jak2 cells in their response to GH. Importantly, MG132 severely inhibited GHR degradation after GH treatment. As expected, MG132 inhibited also constitutive GHR endocytosis (Fig. 5B). We conclude that both constitutive and GH-induced GHR endocytosis are ubiquitin-system dependent. Then we asked whether GH-induced GHR endocytosis also occurs via clathrin-coated pits. We depleted γ2A_GHR_Jak2 cells for clathrin, treated the cells with GH and compared the percentage of change in mature GHR levels between control and clathrin-depleted cells. Fig. 5C clearly shows that clathrin depletion inhibited GH-induced GHR endocytosis, demonstrating that GH-induced GHR endocytosis is clathrin-dependent. Subsequently, we asked whether GH-induced GHR endocytosis depends on the same E3 ligase as constitutive GHR endocytosis. We depleted γ2A_GHR_Jak2 cells for βTrCP, treated the cells with GH

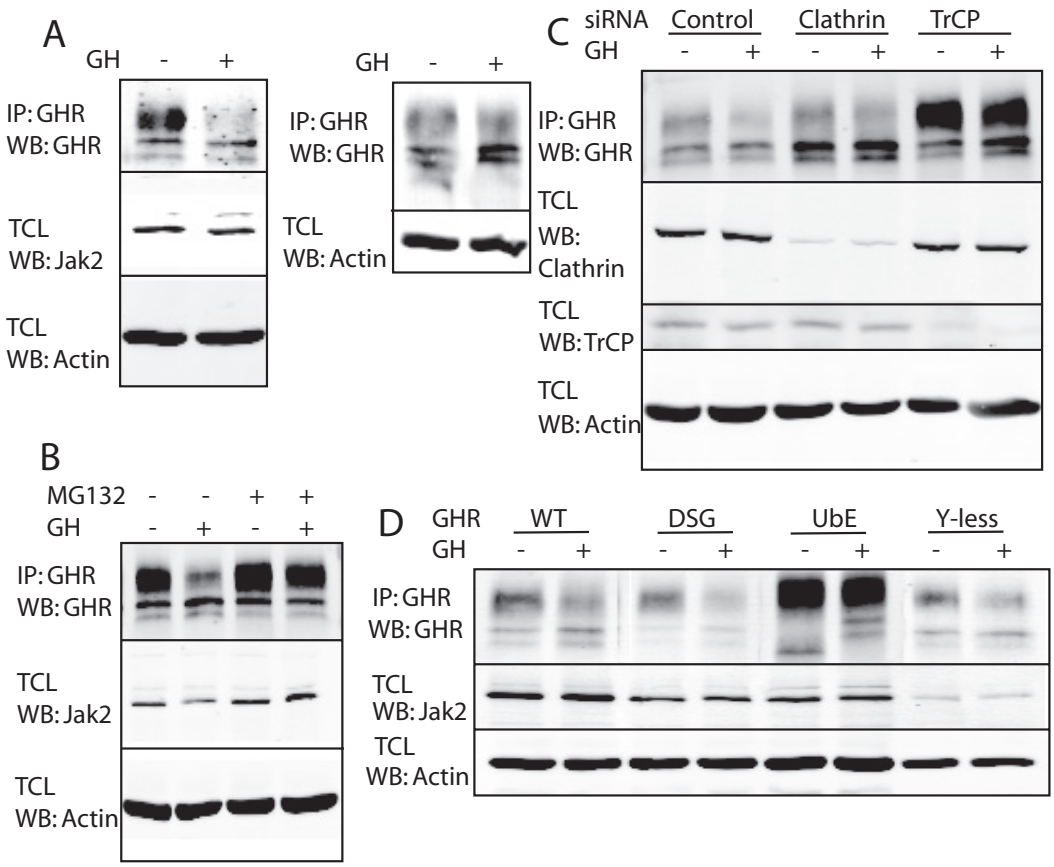

Fig. 5. GH-induced and constitutive GHR endocytosis share the same characteristics.
A. γ2A_GHR_Jak2 cells (left panel) and γ2A_GHR cells (right panel) were stimulated with 500ng/ml GH for 90 minutes as indicated. Total cell lysates (TCL) (lower panels) and GHR immunoprecipitates (upper panels) were analysed on western blot with the indicated antibodies. B. γ2A_GHR_Jak2 cells were pretreated with MG132 for 30 minutes, after which 500ng/ml GH was added to the cells for 90 minutes as indicated. Total cell lysates (TCL) (lower panels) and GHR immunoprecipitates (upper panel) were analysed on western blot with the indicated antibodies. C. γ2A_GHR_Jak2 cells were transfected with control siRNA (lane 1 and 2), clathrin heavy chain siRNA (lanes 3 and 4) or TrCP siRNA (lane 5 and 6). Cells were stimulated with 500ng/ml GH for 90 minutes as indicated, after which total cell lysates (TCL) (lower panels) and GHR immunoprecipitates (upper panel) were analysed on western blot with the indicated antibodies. D. γ2A_Jak2 cells, stably transfected with wild type GHR (WT), GHR mutated in its DSG motif (DSG), GHR mutated in its UbE motif (UbE) or GHR in which all cytosolic tyrosines are mutated into phenylalanines (Y-less) were stimulated with 500ng/ml GH as indicated, after which total cell lysates (TCL) (lower panels) and GHR immunoprecipitates (upper panel) were analysed on western blot with the indicated antibodies. Data in A and C are representatives of three experiments. Data in B and D are representatives of two experiments.

and compared control and βTrCP-depleted cells. βTrCP depletion resulted in a strong inhibition of GHR degradation for both constitutive and GH-induced GHR endocytosis (Fig. 5C). Quantification showed that GHR degradation after GH stimulation is strongly reduced, indicating that βTrCP is required for GH-induced GHR endocytosis.

Previous data from our lab indicated that βTrCP uses the non-canonical UbE motif for constitutive GHR endocytosis, although the canonical DSG motif is present in the GHR cytosolic tail. The question whether the DSG motif is involved in GH-induced endocytosis is relevant, because in case of Prl-induced PrlR degradation, the DSGxxS motif acts as endocytosis motif (19). To clarify this point we used UbE and DSGxxS GHR mutant

cell lines, derived from the γ2A cells expressing low numbers of Jak2. After treating the cells with GH, we observed that mutation of the UbE motif resulted in inhibition of GH-induced GHR degradation (Fig. 5D), indicating that the UbE motif is essential for GH-induced GHR endocytosis. Mutating the DSGxxS motif did not inhibit GH-induced GHR degradation (Fig. 5D). We conclude that both GH-induced GHR endocytosis and constitutive GHR endocytosis depend on βTrCP binding via the UbE-motif rather than via the DSGxxS motif.

The phosphorylated state of tyrosines in the GHR cytosolic tail may play a role in the detachment of phosphorylated Jak2 and may recruit different proteins in GH-induced endocytosis as compared to Jak2-independent endocytosis. We used a γ2A cell line expressing low amounts of Jak2 and a mutant GHR in which all tyrosine residues were replaced by phenylalanine residues (GHRYless). Fig. 5D shows that mutation of all tyrosines in the cytosolic tail of the GHR did not inhibit GH-induced GHR endocytosis.

In summary, we found that GH-induced GHR endocytosis and constitutive GHR endocytosis share the same characteristics. Both pathways are ubiquitin system-, clathrin- and βTrCP-dependent and use the same motif for GHR endocytosis. Moreover, GHR tyrosine residues seem not to be involved. These data strongly suggest that the two pathways are similar.

Relative high Jak2 levels compared to βTrCP correlate with high GH sensitivity of cells

The data presented in this study imply that both βTrCP2 and Jak2 bind to membrane-proximal linear motifs of the GHR at the cell surface. Therefore, relative expression of levels of these two proteins might be important determinants for the endocytosis of GHR and possibly other cytokine receptors. If cells have relatively high Jak2 levels compared to βTrCP2, Jak2-dependent cytokine receptors will be stabilized at the plasma membrane. Conversely, relatively low Jak2 levels will result mainly in constitutive endocytosis. We tested three different cell lines for RNA and protein levels of Jak2 and βTrCP2: IM9 cells that completely depend on GH for GHR endocytosis, and HEK293 and HepG2 cells that exhibit mainly constitutive endocytosis of exogenous GHRs. Using qRT-PCR we found that the Jak2 RNA level relative to the βTrCP2 level is considerably higher in IM9 cells compared to HEK293 and HepG2 cells (Fig. 6A). In addition, the protein levels of Jak2 and βTrCP2 show similar ratios (Fig. 6B). These data suggest that relative high Jak2 levels keep the GHR at the cell surface, rendering cells very GH sensitive. GH stimulation abrogates Jak2 binding to the GHR, resulting in GH-induced GHR endocytosis. On the other hand, relatively high βTrCP2 levels result in more extensive constitutive endocytosis.

Discussion

Previous studies on cytokine receptors showed that Jak family members are able to stabilise GHR, Epo and Tpo receptors in addition to the IFNAR1 and the oncostatin M receptor (5, 8, 13, 15, 28). For Epo receptor it has been shown that Jak2 binding in the ER induces proper folding and efficient trafficking to the cell surface (15). Studies on Tpo receptor describe that Jak2 stabilises the mature Tpo receptor on the cell surface without affecting maturation kinetics (28). Whereas Jak2 does seem to affect neither Tpo nor Epo receptor endocytosis, Jak2 clearly inhibits GHR endocytosis. The different effects of Jaks

A

Cell line	Ratio RNA levels Jak2/β-TrCP2
IM9	3.71 ± 1.03
HEK293	0.75 ± 0.23
HepG2	0.78 ± 0.24

B

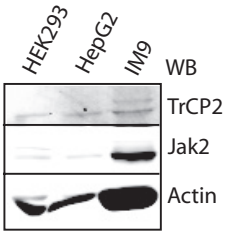

Fig. 6. RNA and protein levels of Jak2, and βTrCP2 in IM9,
HEK293 and HepG2 cells. A. RNA levels were measured using qRT-PCR. Ratios of Jak2/βTrCP2 are displayed of IM9, HEK293 and HepG2 cells as mean values of six experiments ± SD. B. Lysates were prepared of IM9, HEK293 and HepG2 cells and protein levels of Jak2 and βTrCP2 were measured on western blot.

on cytokine receptors suggest that cytokine receptors constitute unique features in their cytoplasmic tails to be differentially affected. Whether a common theme will emerge, i.e. that the kinases stabilise their cognate receptors until receptor activation, remains to be investigated. In summary, the data of our study indicate the following scenario: Jak2 binding prevents endocytosis until GH binding triggers Jak2 phosphorylation and dissociation. GHRs, synthesized in excess to uncommitted Jak2 are available for constitutive βTrCP-mediated endocytosis. Importantly, the mechanism of endocytosis is identical and requires βTrCP. Thus, the GHR depends on GH for endocytosis only if Jak2 is bound.

For the interferon α/β receptor, subunit of the type I interferon receptor (IFNARI), and PrlRs, ligand-induced phosphorylation of a DSGxxS motif C-terminal of box-2 triggers endocytosis and degradation (17, 19). Although such a conserved motif is also present in GHR, we showed that it is not involved in GH-induced endocytosis. The present study demonstrates that the UbE motif is essential for both GH-induced and constitutive GHR endocytosis. In addition, we find that the tyrosines of the GHR are not required for GHR endocytosis. This is in contrast to data by Deng *et al* (5), who showed that the GHR cytosolic tyrosines are essential for GH-induced GHR endocytosis. Since it is very important to express Jak2 at a certain level to be able to measure GHR endocytosis, a different Jak2 expression level in our model system compared to the model system of Deng *et al* could explain the different findings. However, both conclusions fit our model. GHR phosphorylation could facilitate Jak2 dissociation, but GHR phosphorylation definitely does not need a role in our model.

In addition to phosphorylation of Y119 of Jak2, cells must have other mechanisms available to respond to exogenous factors that also induce Jak2 detachment in order to decrease GH sensitivity. These scenarios are required when stresses impose survival mechanisms. In these cases, stress-driven signal transduction pathways might activate Jak2 to abrogate the GHR-Jak2 interaction and induce rapid endocytosis via SCF(βTrCP). Alternatively, a protein that interferes with the Jak2-GHR interaction could be upregulated. No such factor has been identified for the Jak2-GHR interaction yet.

Our findings implicate that, in addition to the expression level of GHR, the cellular concentration of free Jak2 is a major factor that regulates the number of GHRs at the cell surface. A simple explanation would be that the N-terminal half of Jak2, bound to box-1, is sufficient to shield other functional motifs like the UbE sequence, or prevent SCF(βTrCP) complex assembly. Evidence for this scenario comes from a study from Pelletier *et* al, in which they show that both box-1 and the UbE-motif are involved in Jak2 binding and

activation (21). Whether *both* box-1 positions in the dimerised GHR must be occupied remains to be solved. As βTrCP acts as a dimer and possibly needs two UbE motifs for productive binding, it might well be that one Jak2 molecule on a GHR dimer is sufficient to block endocytosis (9, 32).

As many genes have sexually dimorphic expression dependent on either male pulsatile or female continuous patterns of GH secretion (27), our model predicts that cellular Jak2 levels might contribute to this; cells with high Jak2 levels and low GHR expression, like IM9, respond mainly to GH spikes, because they lose their GHRs temporarily, while liver cells with low Jak2 levels and high GHR expression are continuously responsive for both types of GH secretory patterns.

Finally, we propose that GH sensitivity at the cell surface of different cell types is regulated by a delicate balance of Jak2 and βTrCP activities.

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References

1. Haan, C., Kreis, S., Margue, C., and Behrmann, I. (2006) *Biochem Pharmacol* **72**, 1538-1546
2. Ihle, J. N. (1995) *Nature* **377**, 591-594.
3. Hamada, K., Shimizu, T., Matsui, T., Tsukita, S., and Hakoshima, T. (2000) *EMBO J* **19**, 4449-4462
4. Huang, L. J., Constantinescu, S. N., and Lodish, H. F. (2001) *Mol Cell* **8**, 1327-1338
5. Gauzzi, M. C., Barbieri, G., Richter, M. F., Uze, G., Ling, L., Fellous, M., and Pellegrini, S. (1997) *Proc Natl Acad Sci USA* **94**, 11839-11844
6. Radtke, S., Haan, S., Jorissen, A., Hermanns, H. M., Diefenbach, S., Smyczek, T., Schmitz-Vandeleur, H., Heinrich, P. C., Behrmann, I., and Haan, C. (2005) *J Biol Chem* **280**, 25760-25768
7. Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G., and Pellegrini, S. (2003) *EMBO J* **22**, 537-547
8. Hilkens, C. M., Is'harc, H., Lillemeier, B. F., Strobl, B., Bates, P. A., Behrmann, I., and Kerr, I. M. (2001) *FEBS Lett* **505**, 87-91
9. He, K., Loesch, K., Cowan, J. W., Li, X., Deng, L., Wang, X., Jiang, J., and Frank, S. J. (2005) *Endocrinology* **146**, 4755-4765
10. Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999) *Tr Endocrin Metab* **10**, 18-23
11. Argetsinger, L. S., Campbell, G. S., Yang, X. N., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N., and Carter Su, C. (1993) *Cell* **74**, 237-244
12. Lanning, N. J., and Carter-Su, C. (2006) *Rev Endocr Metab Disord* **7**, 225-235
13. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) *EMBO J.* **15**, 3806-3812
14. van Kerkhof, P., Alves dos Santos, C. M., Sachse, M., Klumperman, J., Bu, G., and Strous, G. J. (2001) *Mol Biol Cell* **12**, 2556-2566.
15. van Kerkhof, P., Putters, J., and Strous, G. J. (2007) *J. Biol. Chem.* **282**, 20475-20483
16. Gent, J., van Kerkhof, P., Roza, M., Bu, G., and Strous, G. J. (2002) *Proc Nat Acad Sci USA* **99**, 9858-9863
17. Waters, M. J., Hoang, H. N., Fairlie, D. P., Pelekanos, R. A., and Brown, R. J. (2006) *J Mol Endocrinol* **36**, 1-7
18. Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J., and Constantinescu, S. N. (2005) *J. Biol. Chem.* **280**, 27251-27261
19. Amit, T., Youdim, M. B. H., and Hochberg, Z. (2000) *J Clin Endocrin Metab* **85**, 927-932
20. Funakoshi-Tago, M., Pelletier, S., Matsuda, T., Parganas, E., and Ihle, J. N. (2006) *EMBO J* **25**, 4763-4772
21. Strous, G. J., van Kerkhof, P., Govers, R., Rotwein, P., and Schwartz, A. L. (1997) *J. Biol. Chem.* **272**, 40-43
22. Behrmann, I., Smyczek, T., Heinrich, P. C., Schmitz-Van de Leur, H., Komyod, W., Giese, B., Muller-Newen, G., Haan, S., and Haan, C. (2004) *J. Biol. Chem.* **279**, 35486-35493
23. Pfaffl, M. W., Georgieva, T. M., Georgiev, I. P., Ontsouka, E., Hageleit, M., and Blum, J. W. (2002) *Domest Anim Endocrinol* **22**, 91-102
24. Deng, L., He, K., Wang, X., Yang, N., Thangavel, C., Jiang, J., Fuchs, S. Y., and Frank, S. J. (2007) *Mol Endocrinol* **21**, 1537-1551
25. van Kerkhof, P., Govers, R., Alves dosSantos, C. M. A., and Strous, G. J. (2000) *J Biol Chem* **275**, 1575-1580
26. Petroski, M. D., and Deshaies, R. J. (2005) *Nat Rev Mol Cell Biol* **6**, 9-20
27. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23**, 2028-2036
28. Harper, J. W., Burton, J. L., and Solomon, M. J. (2002) *Genes Dev* **16**, 2179-2206
29. Pickart, C. M., and Fushman, D. (2004) *Curr Opin Chem Biol* **8**, 610-616
30. Pelletier, S., Gingras, S., Funakoshi-Tago, M., Howell, S., and Ihle, J. N. (2006) *Mol Biol Cell* **26**, 8527-8538
31. Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H., and Mcgraw, T. E. (1999) *Proceedings of the National Academy of Sciences of the United States of America* **96**, 6775-6780
32. Li, Y., Suresh Kumar, K. G., Tang, W., Spiegelman, V. S., and Fuchs, S. Y. (2004) *Mol. Cell. Biol.* **24**, 4038-4048
33. Kumar, K. G., Tang, W., Ravindranath, A. K., Clark, W. A., Croze, E., and Fuchs, S. Y. (2003) *EMBO J* **22**, 5480-5490
34. Pelletier, S., Gingras, S., Funakoshi-Tago, M., Howell, S., and Ihle, J. N. (2006) *Mol Cell Biol* **26**, 8527-8538
35. Suzuki, H., Chiba, T., Suzuki, T., Fujita, T., Ikenoue, T., Omata, M., Furuichi, K., Shikama, H., and Tanaka, K. (2000) *J. Biol. Chem.* **275**, 2877-2884
36. Rinn, J. L., and Snyder, M. (2005) *Trends Genet* **21**, 298-305

CHAPTER IV

Specificity, location and function of βTrCP isoforms and splice variants

Joyce Putters, Johan A. Slotman, Jan P. Gerlach and Ger J. Strous

Department of Cell Biology and Institute of Biomembranes
University Medical Center Utrecht

Manuscript submitted

Abstract

$SCF^{\beta TrCP}$ is the ubiquitin ligase for a wide variety of substrates and functions in many processes within cells. βTrCP, the substrate binding factor of the SCF complex, has two isoforms, produced from different genes, and several splice variants. Despite a certain level of redundancy, knock-out studies show different phenotypes indicating different preferential substrates for the two isoforms. However, until now studies with exogenous βTrCP were not able to identify functional differences between βTrCP1 and 2 at the protein level. We generated isoform-specific antibodies against βTrCP to characterise endogenous βTrCP isoforms and splice variants. We show that endogenous βTrCP1 and 2 localise to both nucleus and cytosol. Interestingly, we find that one splice variant of βTrCP2 localises exclusively to the nucleus and another only to the cytosol. In addition, we show that the substrate binding domain of βTrCP is the dominant localisation determinant.

Introduction

Protein ubiquitination affects the fate of target proteins in many essential processes in the cell. The substrate specificity factors of the ubiquitin proteasome system, the ubiquitin ligases, bind to their substrates and transfer ubiquitin directly or indirectly to their target proteins (1). The largest class of ubiquitin ligases are the Skp1-Cullin-F-box protein (SCF) and SCF-like complexes. F-box proteins bind to S-phase-kinase-associated-protein-1 (Skp1) via the N-terminal F-box domain and to their substrates via a C-terminal binding domain (2).

β-transducing-repeat-containing protein (βTrCP) belongs to the family of F-box proteins and was first identified as an interaction partner of the Human Immunodeficiency Virus-1 (HIV-1) Vpu protein (3). As the F-box protein component of a SCF ubiquitin ligase complex, βTrCP is involved in major regulatory mechanisms such as cell cycle progression, metabolism, development, and immunity. Human cells contain two βTrCP proteins, βTrCP1 encoded by BTRC, and βTrCP2 encoded by FBXW11, also known as HOS or βTRCP2 (1,4). The E3 substrate binding domain of βTrCP is the WD40 domain repeat motif, which contains seven WD40 repeats that are structured into a seven-bladed propeller (1). For Vpu, it has been shown that all repeats are required for optimal substrate binding (5).

The cullin proteins (numbered 1-5, and 7) act as molecular scaffolds in the SCF complexes and bind with their N-terminus to Skp1 and with their C-terminus to RING-finger protein Rbx1 and to a specific ubiquitin conjugating enzyme (E2) (6). In case of the interaction between the SCF-complex and the E2 Cdc34, the rapid assembly and disassembly of the E2-E3 complex facilitates ubiquitin chain synthesis. Moreover, residues important for the dynamics of the complex are conserved, suggesting that all SCF complexes and their E2's have the same mechanism of chain assembly (7).

A large number of studies showed that the structural requirements for substrate recognition is not overly stringent, and conforms to a linear motif of 6-9 amino acids starting with two acidic amino acid residues followed by a small amino acid and another acidic moiety in the last position. In β-catenin and IκB (inhibitor of NFκB), the second and last positions constitute phosphorylated serine residues. Upstream of this motif one or several lysine residues serve as ubiquitin acceptors (8-10).

The F-box and WD40 domains of the two isoforms, βTrCP1 and βTrCP2, are highly identical, 86 and 75%, respectively (11,12). Moreover, their WD40 domains show very similar electrostatic surface properties, with a conservation of the central groove covered by positively charged amino acids that interact with the negatively charged residues in the destruction motif of target proteins (13). Indeed, many studies show that the βTrCP isoforms are functionally redundant. This has been reported for the degradation of Wee1, IκB, Per1 and the RE1-silencing transcription factor (Rest) (14-17). Conversely, specificity exists for the early mitotic inhibitor 1 (Emi1) that explicitly requires βTrCP1 for its degradation (18,19). In addition, genetic evidence from gene knock-out experiments in mice shows that βTrCP2 is essential in early development while deletion of βTrCP1 does not affect viability but impairs spermatogenesis and reduces fertility (18). This implies that, although both isoforms occur in all or most cells, they have marked features that cause these differences. The phenotypes may arise from differences in local concentrations, substrate binding, turnover, or expression. However, all these parameters

are inferred from structural differences in the proteins. Therefore, we have investigated the different βTrCP isoforms and their splice variants. In humans, two splice variants of βTrCP1, α and β (without the second exon), and three of βTrCP2, α, β, (with an alternative second exon) and γ, (without the second exon), are predicted to exist.
In this study, we show for the first time the localisation of endogenous βTrCP1 and 2 isoforms and splice variants using isoform-specific antibodies. We find that βTrCP1 and 2 are both present in the nucleus as well as in the cytosol, whereas a striking difference in localisation between two splice variants of βTrCP2 was observed: while βTrCP2γ resides exclusively in the cytosol, βTrCP2β is solely detectable in the nucleus. We also show that endogenous βTrCP has different binding affinities to β-catenin compared to exogenous βTrCP.

Experimental procedures

Cell culture and transfections

HEK293 cells were cultured in 4.5 g/l glucose (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 0.1 mg.ml streptomycin (both Invitrogen). HEK293 cells, stably expressing the Tet-repressor (HEKTR), were a gift of Dr. Madelon Maurice and were maintained in DMEM with 4.5 g/l glucose, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 12 μg/ml Blasticidin-S (MP Biomedicals). HEKTR cells, stably expressing βTrCP1 or 2 (upon 1 ng/ml doxycyclin addition) (HEKTR_iTrCP), were cultured in the maintenance medium for HEKTR cells, supplemented with 170 μg/ml zeocin (Invitrogen). HEKTR_iTrCP cells that were either stably transfected with GHR (HEKTR_GHR_iTrCP1 or 2), were cultured in HEKTR_iTrCP maintenance medium, supplemented with 600 μg/ml geneticin (Invitrogen).

Transfections, DNA constructs and siRNA

Wild type GHR in pcDNA3.1, GFP siRNA and βTrCP siRNA were described before, as were wild type Flag-βTrCP2, Flag-βTrCP2(ΔWD40), βTrCP2(WD40), βTrCP2(ΔF) and βTrCP2(ΔD) (20). Inducible βTrCP1β and 2α were constructed by cloning βTrCP1β and βTrCP2α into pcDNA4/TO (Invitrogen), Tet-repressor is in pcDNA6 and GST-βTrCP1β (1-93) and GST-βTrCP2α (1-68) are in pGEX1λt. βTrCP1α (1-167) (from clone IRAKp961E0762Q2 obtained from RZPD) and β (1-131) were expressed in a pcDNA4/TO vector. EST clones containing N-terminal fragments of TRCP2 beta and gamma were a gift from Dr. Gabor Gyapay (Genoscope). Myc-β-catenin was a kind gift of Dr. Madelon Maurice. DNA transfections were performed using FuGene6 (Roche) and siRNA transfections with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection of Myc- β-catenin was done using calcium phosphate.

Antibodies and materials

Anti-GHR(B) was described before (21). Anti-actin was purchased from MP Biomedicals, whereas anti-Flag polyclonal and anti-Myc monoclonal were from Sigma. Leptomycin B, used for 12 h at 10 ng/ml, was purchased from Sigma. Cycloheximide was bought from ICN and was used at 100 μg/ml. Doxycyclin was obtained from Clontech. biotin-GH was described before (22).

Antibody generation and affinity purification

GST fusion proteins of residues 1-93 of βTrCP1β and residues 1-68 of βTrCP2α were injected into rabbits and antisera were obtained. We affinity purified the antisera using CNBr activated Sepharose-4 beads (Pharmacia), according to the manufacturer's manual.

Preparation of nuclear, cytosolic and membrane fractions

Subconfluent cells were collected with trypsin-EDTA (Invitrogen) and washed with phosphate-buffered saline (PBS), after which the cells were washed two times in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM Dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinine), after which the cells were incubated in hypotonic buffer on ice for 10 minutes. The cells were dounced 50 times with a B-type pestle, after which the lysates were centrifuged for 5 seconds at 16000xg. The supernatant was fractionated into a cytoplasmic and a membrane fraction by centrifugation at 100,000g for 1 hour. The pellet was washed 2 times with hypotonic buffer and designated as the nuclear fraction.

Immunofluorescence and confocal microscopy

HEKTR_GHR_iTrCP1 or 2 cells were grown on cover slips. The cells were washed and fixed with 3% paraformaldehyde in 0.1 M sodium-phosphate buffer pH 7.4. After washing with PBS, the cells were incubated with 0.02 M NH_4Cl for 5 minutes. Subsequently, cells were incubated with 0.2% TritonX-100 for 5 minutes, followed by three washes with PBS. 0.5% bovine serum albumin (BSA) was added for 15 minutes and the primary antibody in 0.5% BSA was left on the cells for 30 minutes. The cells were washed with 0.5% BSA and incubated with Alexa goat anti-rabbit 488 (Molecular probes) for 30 minutes. After washing with 0.5 mg/ml BSA in PBS and PBS, the cells were mounted with mowiol and analyzed using a confocal microscope.

Cell lysis, immunoprecipitation and biotin-GH pull down

Cell lysates were prepared by washing the cells with cold PBS, after which the cells were lysed with 1% Triton X-100, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF in PBS for 15 minutes. For the turnover experiments, the cell lysates were prepared by incubating the cells in a buffer containing 1% sodium dodecyl sulphate (SDS), 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF in PBS for 10 minutes at 100°C. The lysates were centrifuged for 5 minutes and the supernatants were used for immunoprecipitations by incubation with anti-myc for 2 hours followed by incubation of 45 minutes with protein A agarose beads. Biotin-GH pull down was performed by incubating the lysates for 1 hour with biotin-GH, followed by a 30-minute incubation with streptavidin agarose beads. Lysates, immunoprecipitates and pull down samples were subjected to SDS-PAGE and western blotting, after which the membranes were detected with the indicated antibodies. Western blots were analysed using an Odyssey infrared imaging system and quantified using Odyssey software (Li-Cor Biosciences, Lincoln, NE).

Tissue profiling and RT-PCR

Tissue profiling was done using Clontech Human Multiple Tissue cDNA panels I and II and PCR was done according to the user's manual. The following primers were used:

βTrCP1 forward primer GATCGGATCCATGGACCCGGCCGAGGCG, βTrCP1 reverse primer: GTACGCGGCCGCGTGCCCATGTTGGTAATGAC, βTrCP2 forward primer TTTGGATCCATGGAGCCCGACTCGGTGATTGAGGAC and βTrCP2 reverse primer GTACGCGGCCGCATGACCATGCTGATAATGAC. The same primers were used to perform a RT-PCR for βTrCP1 and 2 on the RNA isolated from HEK293 cells. DNA products were verified by direct sequencing.

Results

Detection of βTrCP1 and 2 isoforms by specific antibodies

In order to identify differences between endogenous βTrCP isoforms and splice variants that might account for their functional differences, we generated isoform-specific antibodies. The schematic structure of βTrCP, depicted in Fig. 1A, shows a variable domain in the N-terminus. Therefore, we selected the variable domains of βTrCP to generate antibodies specific for either βTrCP1 or 2. The splice variants are identifiable by their differences in molecular weight. To test antibody sensitivity and specificity, we prepared nuclear and cytosolic fractions from HEKTR cells and analysed the samples on western blot. Fig. 1B shows expected bands of ~60 kD and 50 kD for endogenous βTrCP1 and 2 respectively, indicating that the antibodies are capable of detecting endogenous βTrCP. To determine whether the observed bands are specific, we depleted the cells for βTrCP with a siRNA probe directed against both βTrCP1 and 2 and a control siRNA (GFP). As shown in Fig. 1B, the bands detected in GFP-silenced cells completely disappeared in βTrCP-silenced cells. Importantly, the band detected by anti-βTrCP1 was not detected by

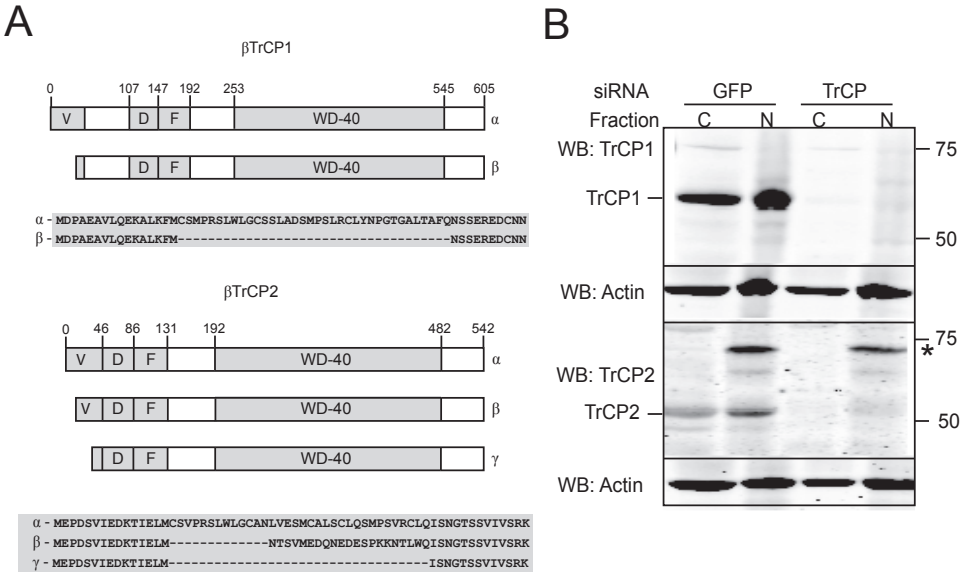

Fig. 1. Characterisation of antibodies against the N-termini of βTrCP1 and 2.
A. Schematic representation of the structural organisation of the isoforms βTrCP1 and 2 and their splice variants. V= Variable domain, D= Dimerisation domain, F= F-box. The variable domains of βTrCP1β and 2α were expressed as GST fusion proteins and used for antibody production in rabbits. B. HEKTR cells were silenced for GFP and βTrCP followed by the preparation of cytosolic and nuclear fractions. Western blot were detected with the indicated antibodies. The asterisk indicates a background band.

anti-βTrCP2 and *vice versa*, which shows that the antibodies do not cross react. With the βTrCP2 antiserum, we detected one abundant background band that was present only in the nuclear fraction, which was not silenced with βTrCP siRNA. These results clearly indicate that the antibodies are specific and can be used to identify differences in cellular localisation, substrate binding and turnover.

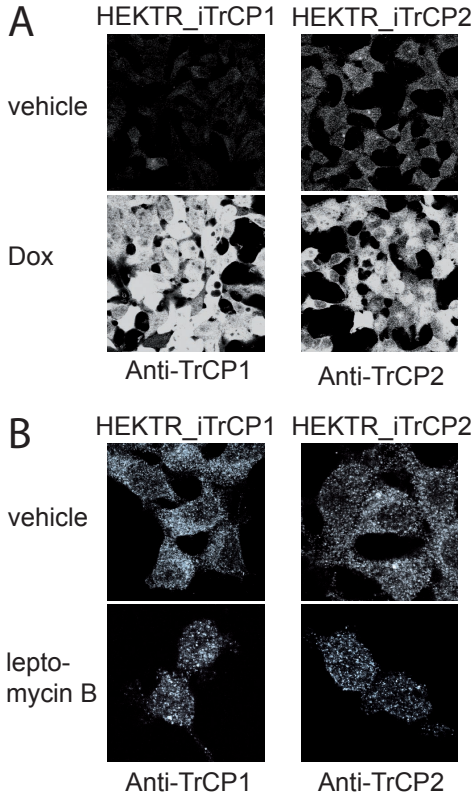

Fig. 2. Both endogenous and exogenous βTrCP1 and 2 localise to both the nucleus and the cytosol
A. HEKTR_iTrCP1 or 2 cells were treated with vehicle or doxycyclin, after which the cells were immunolabelled with affinity-purified anti-βTrCP1 or 2 and visualised by confocal microscopy. B. HEKTR_iTrCP1 or 2 cells were treated overnight with vehicle or 10ng/ml leptomycin B, after which the cells were labelled with affinity-purified anti-βTrCP1 or 2 and visualised by confocal microscopy. Microscopy settings were the same for all pictures.

Endogenous and exogenous βTrCP1 and 2 localise to both nucleus and cytosol

One obvious explanation for substrate-specificity of βTrCP1 and 2 is a different localisation. To localise βTrCP1 and 2 isoforms in the cell, we used HEKTR-cells that express either βTrCP1 or 2 from a doxycyclin-inducible DNA construct. After treatment with doxycyclin, we stained with affinity-purified βTrCP1 and 2 antibodies and visualised the cells using confocal microscopy. Fig. 2A shows that induction of exogenous βTrCP1 and 2 resulted in a strong increase in signal intensity, indicating that the antibodies work after formaldehyde fixation of cells. The vehicle-treated cells show that endogenous βTrCP1 and 2 both localise to both the cytoplasm and the nucleus. The same localisations were detected for exogenous (doxycyclin-induced) βTrCP. Leptomycin B treatment, which results in inhibition of nuclear export via XPO1/CRM1, resulted in localisation of both (endogenous) isoforms in the nucleus (Fig. 2B), indicating that they shuttle between the nucleus and the cytosol. Together, these results show no differences for the localisation of βTrCP1 and 2 isoforms. Both endogenous and exogenous βTrCP isoforms are present in the nucleus and the cytoplasm.

βTrCP splice variants localise differently within the cell

In a previous study with mouse cells, 8 splice variants for both mouse βTrCP1 and 2 were identified (23). To determine the occurrence of βTrCP splice variant mRNAs in human HEKTR-cells, we performed RT-PCR and detected two abundant human splice variants for βTrCP1 (α and β) and two for βTrCP2 (β and γ) (Fig. 3A). For βTrCP1, one extra low abundant splice variant was detected, which we named βTrCP1α'. Interestingly, βTrCP2α RNA, used in most overexpression studies, was not detected in HEKTR cells.

As immunofluorescence was unable to distinguish the different βTrCP splice variants, we performed cell fractionation. In nuclear and cytosolic fractions of doxycycline-inducible HEKTR_iTrCP1 or 2 cells we detected only one endogenous splice variant of βTrCP1, which migrated as exogenous βTrCP1β (Fig. 3B). As the mRNA of both βTrCP1α and β splice variants are produced in HEK293 cells (Fig. 3A) and the βTrCP1 antibody recognises both βTrCP1α and β variants (Fig. 3D), it might be that the difference in molecular weight is too small to distinguish βTrCP1α from β on western blot. Another explanation would be that the protein βTrCP1α is unstable. We conclude from our immunofluorescence data that endogenous βTrCP1 and exogenous βTrCP1β localise to both the cytoplasm and the nucleus.

We detected two endogenous βTrCP2 isoforms, of which the fast-migrating one was present in the cytosol and the slow-migrating one was found in the nucleus. Since the overexpressed βTrCP2 is the α-variant, the endogenous faster-migrating TrCP2 splice variants are very likely the β and γ variants. The absence of endogenous βTrCP2α is supported by the RT-PCR data (Fig. 3A). This is also in agreement with a previous study in mouse tissues, in which it was shown that the most commonly used βTrCP2 splice variant is expressed to a very low extent (23).

Since several cell surface signalling receptors are βTrCP substrates, we investigated whether βTrCP that is outside the nucleus, is membrane-bound or cytosolic. As shown in Fig. 3C and already indicated by the granular appearance in Fig. 2B, endogenous βTrCP1 and 2 are mainly attached to membranes rather than existing free in the cytosol. In contrast, membrane-bound and cytosolic exogenous βTrCP1 and 2 levels are similar, probably because the substrates at the plasma membranes are saturated with βTrCP because of the high levels of overexpressed βTrCP.

Immunocytochemical data suggest that βTrCP1 is mainly responsible for ubiquitination of nuclear substrates, including ATF4 and Emi1 (19,24), while βTrCP2 is also involved in cell surface receptor degradation (20,25). Since βTrCP2α localises to both nucleus and cytosol, we chose this variant to define a localisation motif or domain and constructed several Flag-tagged βTrCP mutants: lacking either the F-box (ΔF), the dimerization domain (ΔD) or the WD40 domain (ΔWD40) or only the WD40 domain (WD40). Fig. 3E clearly shows that wild type βTrCP2α is highly abundant in the nucleus compared to the cytosol. In contrast, the ΔWD40 mutant shows an equal distribution between nucleus and cytosol, demonstrating that the WD40 domain is important for nuclear localisation. As wildtype βTrCP, the WD40 domain itself localised to the nucleus. In fact, all mutant βTrCP species with an intact WD40 domain distributed equally supporting the notion that substrate binding is important for βTrCP localisation.

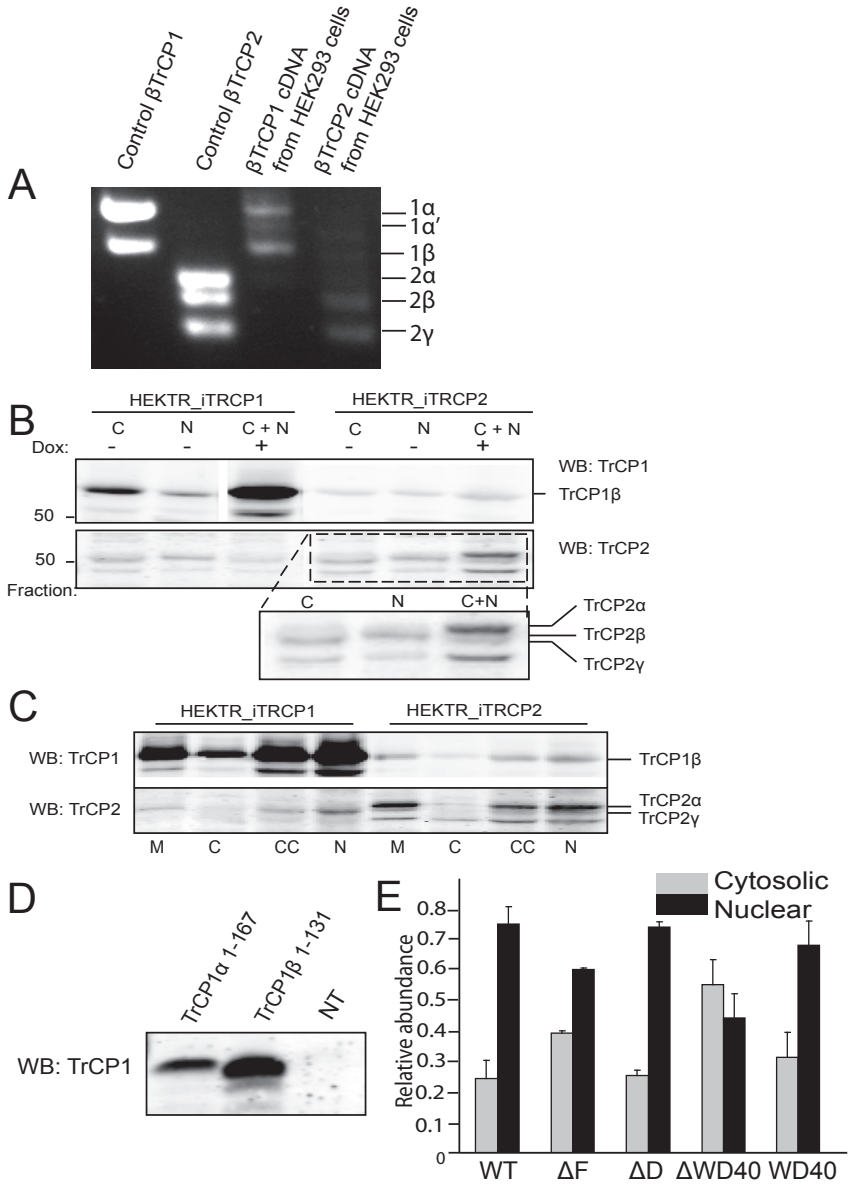

Fig. 3. Expression pattern and cellular localisation of βTrCP2 splice variants.
A. RNA was isolated from HEK293 cells and a RT-PCR with primers specific for either βTrCP1 or 2 was performed. DNA from known splice variants was used as positive control. B. HEKTR_iTrCP1 and HEKTR_iTrCP2 cells were treated with and without doxycyclin followed by the preparation of cytosolic (C) and nuclear (N) fractions. The fractions were analysed on western blot using affinity-purified anti-βTrCP1 or 2. On the right, the splice variants are indicated. C. HEKTR_iTrCP1 and HEKTR_iTrCP2 cells were treated with doxycyclin followed by the preparation of nuclear (N) and crude cytosolic (CC) fractions. The CC fractions were further separated into cytosolic (C) and membrane (M) fractions. The fractions were analysed as in Fig. 3B. On the right, the splice variants are indicated. The experiments are shown as typical examples of three independently performed. D. βTrCP1α 1-167 and βTrCP1β 1-131 were expressed in HEKTR cells and detected with anti-βTrCP1. E. Flag-tagged βTrCP mutants were expressed in HEKTR cells followed by the preparation of cytosolic and nuclear fractions. The fractions were analysed on western blot using anti-Flag followed by quantification using Odyssey software. Data are displayed as mean values of three experiments ± SD.

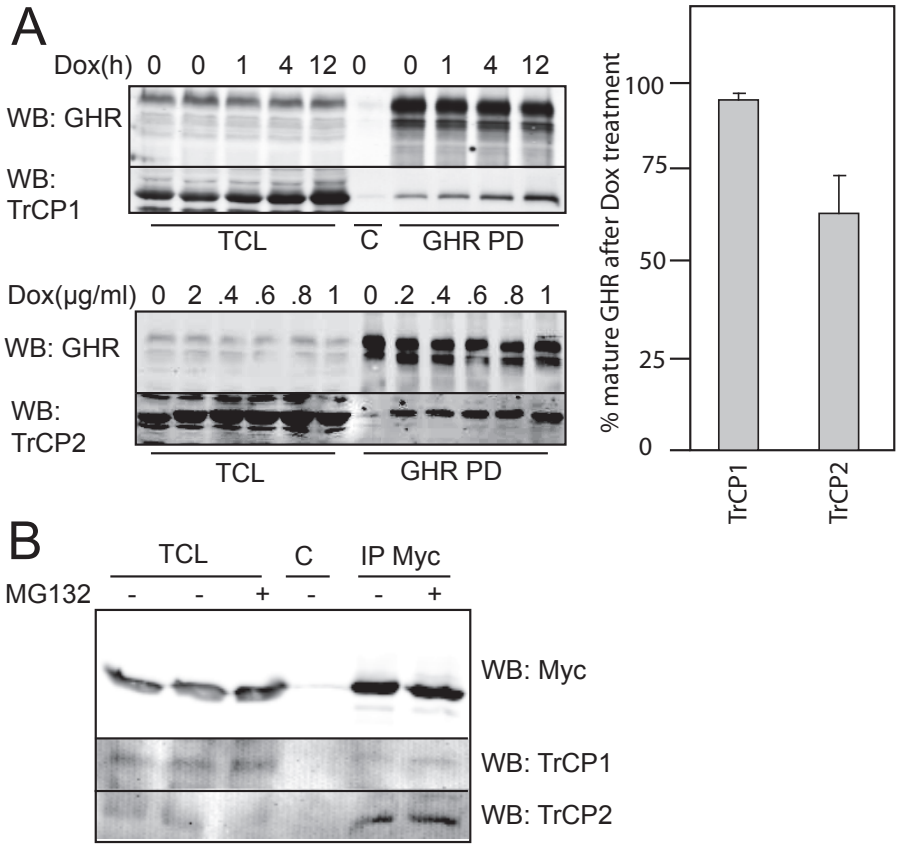

Fig. 4. Binding characteristics of βTrCP1 and 2 for GHR and β-catenin.
A. To obtain increasing levels of exogenous βTrCP, cells were treated with doxycyclin in a time course (TrCP1) with 1 ng/ml doxycycline or a doxycyclin concentration range (βTrCP2) for 12 hours to obtain increasing levels of exogenous βTrCP. The GHR was pulled down using biotinylated GH and co-precipitated βTrCP was analysed on western blot. On the right, mature GHR is quantified after a 12 hour doxycyclin induction of either βTrCP1 or 2. Data are means from three (TrCP1) or seven (TrCP2) experiments ± SD. TLC, total cell lysate; PD, pull-down. B. HEKTR cells were transfected with myc-β-catenin followed by immunoprecipita¬tion with anti-Myc. Coprecipitated endogenous βTrCP1 and 2 were analysed on western blot. C, Control (no biotinylated GH or IgG added).

Substrate binding affinity of βTrCP1 and 2 for GHR and β-catenin

In a previous study we showed that βTrCP is essential for growth hormone receptor (GHR) endocytosis and that despite equal binding, only βTrCP2 is functional (20). As substrate specificity is important to establish functional differences among βTrCP variants towards substrates like IκB, β-catenin and GHR (8,9,20,26), we tested the binding of the endogenous βTrCP isoforms to GHR and β-catenin.

We induced βTrCP1 and 2 at a fixed doxycyclin concentration in a time course (βTrCP1) or by a doxycyclin concentration curve (βTrCP2) and analysed the amount of βTrCP bound to GHR. Fig. 4A shows that GHR binds both endogenous and exogenous βTrCP1 and 2. Comparing input and coprecipitated βTrCP, we conclude that binding affinities were approximately equal. Based on the cytosolic localisation of βTrCP2γ, we expect this splice variant to be essential for GHR endocytosis. βTrCP2 coprecipitation with the GHR shows two βTrCP2 splice variants, of which the fast migrating one is endogenous βTrCP2γ and

the slow migrating variant is exogenous βTrCP2α. Like in Fig. 3B, no βTrCP2α is detected without doxycyclin treatment, which means that the fluorescent signals in Fig. 2B reflect the location of *endogenous* βTrCP1 and 2 and not leakage of the inducible promotor of the βTrCP constructs. As expected, upon induction of βTrCP2 the amount of steady state mature GHR decreased, confirming that βTrCP2 is functional in GHR endocytosis, while upregulation of βTrCP1 did not affect mature GHR levels (Fig. 4A, histogram) (20).
A redundant role of mammalian βTrCP1 and 2 in ubiquitination and degradation of β-catenin was proposed on the basis of RNAi analysis and data from mice with genetic ablation of βTrCP1 (18). To examine whether βcatenin binding to endogenous βTrCP is in line with these findings, we analysed co-immunoprecipitations of myc-tagged β-catenin and βTrCP (Fig. 4B). Independent of proteasomal activity, we observed relatively more endogenous βTrCP2 than 1 coprecipitating with β-catenin, comparing input and coprecipitated βTrCP. This experiment shows that β-catenin interacts preferentially with βTrCP2. Whether that is due to different localisation or affinity remains to be determined. We conclude that the GHR does not preferentially bind a particular βTrCP isoform, whereas β-catenin binds preferably (endogenous) βTrCP2.

Both βTrCP1 and 2 are stable proteins

The stability of ubiquitin ligases is an important parameter in major regulatory processes. βTrCP2 is stabilized in the presence of phosphorylated substrates (27). To determine the stability of endogenous βTrCP1 and 2, we treated HEKTR cells with cycloheximide and quantified endogenous βTrCP1 and 2 levels after different chase periods. Fig. 5 clearly shows that the turnover of both βTrCP1 and 2 is low, since after four and a half hours not even half of the proteins have been degraded. We conclude that the half lives of endogenous βTrCP1 and 2 isoforms are similar, demonstrating that the turnover does not contribute to their differential cellular function.

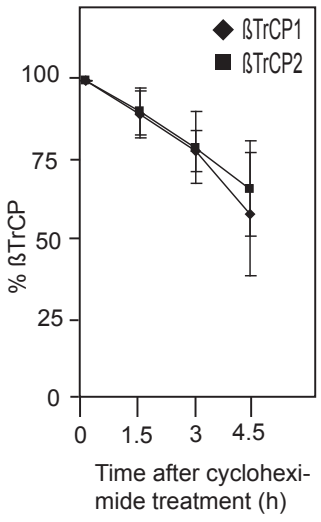

Fig. 5. Endogenous βTrCP1 and 2 have relatively long half lives. HEKTR cells were treated with cycloheximide and lysed after the indicated time points. βTrCP1 and 2 levels were analysed on western blot and quantified using Odyssey software. Data are means of four independent experiments ± SD.

Tissue distribution of RNA levels of splice variants of βTrCP1 and 2

Based on the finding that splice variants localise differently in cells, we speculated that different cell types use differential alternative splicing to regulate the level of specific variants of βTrCP in cells. Therefore, we performed a RT-PCR on a variety of tissues and determined whether their mRNA levels varied among these tissues. Fig. 6A shows that three mRNAs of βTrCP1 were detectable instead of the predicted two, just like we observed in Fig. 3A. These data are in agreement with a study from Seo *et al* (23). Out of the three detected mRNAs, two are very abundant in several tissues (α and β), whereas the α' variant is predominantly expressed in testis. βTrCP1 mRNA levels vary considerably in different tissues (Fig. 6A), so it might be that different cell types do not equally depend on the various splice variants. For βTrCP2, we detected four mRNA splice variants; β, γ and two splice variants indicated with asterixes (Fig. 6B). The mRNAs of these two splice variants were also detected by Seo *et al*, but we did not detect either of them at the protein level, suggesting that the proteins are highly unstable. Although the signals were low for βTrCP2β and γ (Fig. 6B), the RNA expression patterns of the tested tissues seem to differ, suggesting that regulation of βTrCP2 mRNA splicing might be a mechanism for cells to regulate βTrCP2 splice variant levels.

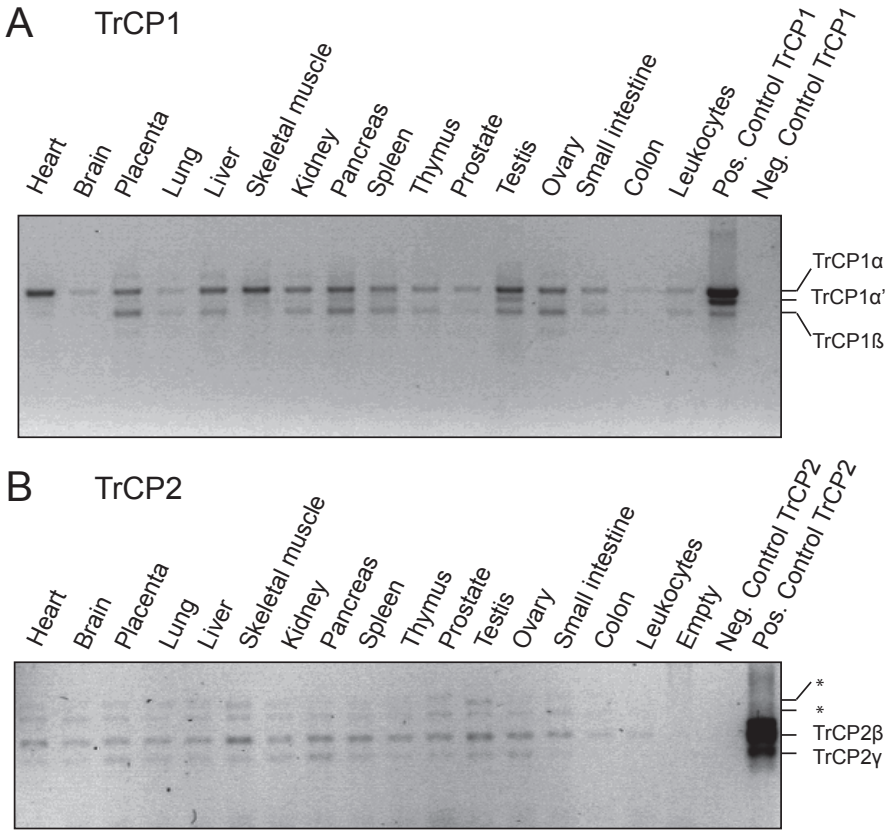

Fig. 6. Tissue profiling of mRNA levels of βTrCP1 and 2.

RNA tissue profiling for βTrCP1 (A) and βTrCP2 (B). Asterisks indictated in B show two βTrCP2 splice variants in addition to the β and γ variants that were not detected on the protein level. Data are representive of two independent experiments.

Discussion

The importance of $SCF^{\beta TrCP}$ is reflected by its involvement in key processes in the cell, like the NFκB and the Wnt signalling pathway. Many studies have been performed in order to identify which βTrCP isoform acts on a given substrate. Because of the lack of antibodies recognizing endogenous βTrCP, these studies were all done with exogenous βTrCP, in particular βTrCP1β and 2α. Overexpression of a protein might result in aberrant localisation, substrate binding characteristics or half life compared to the endogenous protein. As a consequence of using exogenous βTrCP, most studies investigated only one particular splice variant. Our isoform-specific antibodies enabled a precise study of localisation, substrate affinity and turnover of endogenous βTrCPs.

We found that both endogenous βTrCP1 and 2 isoforms are localised in the nucleus as well as in the cytosol. However, whereas endogenous βTrCP2β was present exclusively in the nucleus, endogenous βTrCP2γ was found only in the cytosol. Moreover, we showed that the substrate binding domain is an important factor for nuclear localisation. However, in case of the different location of βTrCP2β and 2γ this cannot be contributed to their (identical) WD40 domain. Very likely, the variable N-termini might play a role as well. Both endogenous βTrCP1 and 2 isoforms that are located outside the nucleus, are mainly membrane-localised. As the substrate binding domain appears the dominant localisation factor, it is tempting to speculate that most of the processes outside the nucleus that involve βTrCP occur membrane-associated. In contrast, exogenous βTrCP is distributed equally over cytosol and membrane fractions, indicating that high βTrCP expression levels saturate the βTrCP substrates located at the membrane with βTrCP. Excess βTrCP that does not encounter a substrate on the membrane will mislocalise in the cytosol.

The identified differences in the localisation of βTrCP isoforms and splice variants suggest that their regulation is important, especially since βTrCP binds to some substrates concentration-dependently instead of recognising phosphorylated DSGXXS motif. This principle of concentration-dependent βTrCP binding implies that uncontrolled βTrCP expression might result in the development of tumours, because many βTrCP substrates are key players in the regulation of many cellular processes that are related to cell division and cancer (28). In GHR, βTrCP binds to the endocytosis motif (DDSWVEFILDIDD) without any modifications and its ligase activity probably depends mainly on the cytosolic concentration of the βTrCP2γ variant. GH stimulation of the GHR results in an elevated Insulin-like Growth Factor-I (IGF-I) level, which has been associated with a number of cancers. IGF-I levels can be reduced by blocking GH-binding to GHR or by upregulation of βTrCP2γ (29). The tumour suppressor TAp63γ also seems to bind βTrCP without upstream action of a kinase. In contrast to many other substrates, βTrCP binding stabilises TAp63γ, resulting in G1 cell cycle arrest (30). In such conditions, uncontrolled βTrCP expression could result in improper regulation of the cell cycle and cancer.

Despite the high substrate diversity, it has been suggested that βTrCP is mainly tumour-promoting and is considered an oncogene. This is reflected by an increased expression of βTrCP in a variety of cancers, whereas mutations in βTrCP are rarely found in human tumours and primary cancers. A striking example is hepatocellular carcinomas, which show constitutive NFκB activation together with high βTrCP levels (30-32). In addition, some cancers are specifically associated with one of the βTrCP isoforms, like βTrCP1 in hepatoblastomas and colectoral cancers and βTrCP2 in breast/gastric and prostate cancers.

Fig. 6 shows that different cell types have differences in βTrCP splice variant mRNAs, suggesting that also differential expression of βTrCP splice variants might be relevant for the development of cancers. Not only expression of the functional, but also of the non-functional βTrCP splice variants is important for a given substrate. βTrCP variants that bind to, but are not functional towards a given substrate might act as dominant-negative. Another way of regulating βTrCP isoform levels might be binding of the WD40 domain of βTrCP1 and not 2 to pseudosubstrate hnRNP-U (33). Possibly, more proteins with a similar function are expressed in cells. In addition, proteins can regulate βTrCP expression in cells, which has been shown for BRAF in human melanoma cells (30).
βTrCP acts within the SCF complex and requires the other SCF components for its activity. The high levels of βTrCP observed in tumours suggest that βTrCP is the limiting factor for activity of the SCF complex. This idea is supported by the fact that βTrCP overexpression in cells often results in accelerated degradation of its substrates, indicating that the other SCF components are not limiting.
Together, we determined the localisation, turnover and binding affinity for GHR and β-catenin of endogenous βTrCP1 and 2 isoforms and their splice variants. We conclude that especially localisation and substrate binding affinity are different between βTrCP variants, suggesting that these parameters are important for their function. The presented findings on βTrCP will be important for a better understanding of the mechanism of action of βTrCP and its role in cancer.

Acknowledgements

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References

1. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23**, 2028-2036
2. del Pozo, J. C., and Estelle, M. (2000) *Plant Mol Biol* **44**, 123-128
3. Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998) *Mol Cell* **1**, 565-574
4. Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M., and Pagano, M. (1999) *Curr Biol* **9**, 1177-1179
5. Coadou, G., Gharbi-Benarous, J., Megy, S., Bertho, G., Evrard-Todeschi, N., Segeral, E., Benarous, R., and Girault, J. P. (2003) *Biochemistry* **42**, 14741-14751
6. Cardozo, T., and Pagano, M. (2004) *Nat Rev Mol Cell Biol* **5**, 739-751
7. Kleiger, G., Saha, A., Lewis, S., Kuhlman, B., and Deshaies, R. J. (2009) *Cell* **139**, 957-968
8. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev.* **13**, 270-283
9. Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., and Polakis, P. (1999) *Curr Biol* **9**, 207-210
10. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) *Mol Cell* **11**, 1445-1456
11. Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J., and Harper, J. W. (1999) *Curr Biol* **9**, 1180-1182
12. Butticaz, C., Michielin, O., Wyniger, J., Telenti, A., and Rothenberger, S. (2007) *J Virol* **81**, 1502-1505
13. Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Suzuki, T., Ichiyama, A., Ikenoue, T., Omata, M., Furuichi, K., and Tanaka, K. (1999) *Biochem and Biophys Res Comm* **256**, 127-132
14. Suzuki, H., Chiba, T., Suzuki, T., Fujita, T., Ikenoue, T., Omata, M., Furuichi, K., Shikama, H., and Tanaka, K. (2000) *J Biol Chem* **275**, 2877-2884
15. Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Watanabe, N., Hunter, T., and Osada, H. (2004) *PNAS* **101**, 4419-4424
16. Shirogane, T., Jin, J., Ang, X. L., and Harper, J. W. (2005) *J. Biol. Chem.* **280**, 26863-26872
17. Westbrook, T. F., Hu, G., Ang, X. L., Mulligan, P., Pavlova, N. N., Liang, A., Leng, Y., Maehr, R., Shi, Y., Harper, J. W., and Elledge, S. J. (2008) *Nature* **452**, 370-374
18. Guardavaccaro, D., Kudo, Y., Boulaire, J., Barchi, M., Busino, L., Donzelli, M., Margottin-Goguet, F., Jackson, P. K., Yamasaki, L., and Pagano, M. (2003) *Dev Cell* **4**, 799-812
19. Margottin-Goguet, F., Hsu, J. Y., Loktev, A., Hsieh, H. M., Reimann, J. D., and Jackson, P. K. (2003) *Dev Cell* **4**, 813-826
20. van Kerkhof, P., Putters, J., and Strous, G. J. (2007) *J Biol Chem* **282**, 20475-20483
21. van Kerkhof, P., Govers, R., Alves dosSantos, C. M. A., and Strous, G. J. (2000) *J Biol Chem* **275**, 1575-1580
22. van den Eijnden, M. J. M., Lahaye, L. L., and Strous, G. J. (2006) *J Cell Sci* **119**, 3078-3086
23. Seo, E., Kim, H., Kim, R., Yun, S., Kim, M., Han, J. K., Costantini, F., and Jho, E. H. (2009) *Cell Signal* **21**, 43-51
24. Lassot, I., Segeral, E., Berlioz-Torrent, C., Durand, H., Groussin, L., Hai, T., Benarous, R., and Margottin-Goguet, F. (2001) *Mol Cell Biol* **21**, 2192-2202.
25. Li, Y., Suresh Kumar, K. G., Tang, W., Spiegelman, V. S., and Fuchs, S. Y. (2004) *Mol. Cell. Biol.* **24**, 4038-4048
26. Marikawa, Y., and Elinson, R. P. (1998) *Mech Dev* **77**, 75-80
27. Fuchs, S. Y., Chen, A., Xiong, Y., Pan, Z. Q., and Ronai, Z. (1999) *Oncogene* **18**, 2039-2046
28. Frescas, D., and Pagano, M. (2008) *Nat Rev Cancer* **8**, 438-449
29. Khandwala, H. M., McCutcheon, I. E., Flyvbjerg, A., and Friend, K. E. (2000) *Endocr Rev* **21**, 215-244
30. Liu, J., Suresh Kumar, K. G., Yu, D., Molton, S. A., McMahon, M., Herlyn, M., Thomas-Tikhonenko, A., and Fuchs, S. Y. (2007) *Oncogene* **26**, 1954-1958
31. Chiaur, D. S., Murthy, S., Cenciarelli, C., Parks, W., Loda, M., Inghirami, G., Demetrick, D., and Pagano, M. (2000) **88**, 255-258
32. Dhawan, P., and Richmond, A. (2002) *J Biol Chem* **277**, 7920-7928
33. Davis, M., Hatzubai, A., Andersen, J. S., Ben-Shushan, E., Fisher, G. Z., Yaron, A., Bauskin, A., Mercurio, F., Mann, M., and Ben-Neriah, Y. (2002) *Genes Dev.* **16**, 439-451

CHAPTER V

The contribution of tyrosine residues to growth hormone receptor signalling

Joyce Putters and Ger J. Strous

Department of Cell Biology and Institute of Biomembranes
University Medical Center Utrecht

Manuscript in preparation

Abstract

Growth hormone (GH) is not only required for longitudinal growth, it is also involved in protein, fat and carbohydrate metabolism. GH binding results in GHR phosphorylation via Janus kinase 2 (Jak2). Phosphorylated tyrosine residues within the GHR cytosolic tail serve as docking sites for signalling molecules, adapter proteins and signal regulatory proteins. Several *in vitro and in vivo* studies have identified tyrosine residues that are involved in GHR, STAT5B and MAPK phosphorylation, but there is still debate about their specific contributions to the signalling process. In this study, we screened a large number of GHR tyrosine mutants to determine their contribution to the STAT5 and MAPK signalling pathways. In agreement with previous studies, we confirm that tyrosine residues 487, 534 and 627, but not residue 566, are most important for GHR and STAT5 phosphorylation. Finally, we report that mutation of tyrosine 627 results in constitutive GHR, Jak2 and MAPK hyperphosphorylation.

Introduction

Growth hormone (GH) is involved in the regulation of postnatal longitudinal growth, in part by inducing insulin-like growth factor 1 (IGF1) (1). In addition, GH has a regulatory role in cellular protein, fat and carbohydrate metabolism and stimulates differentiation and mitogenesis of cells (2). GH signalling is initiated by the binding of GH to its receptor, the GH receptor (GHR), which results in a con-formational change in the GHR (3). As a consequence, the spatial positions of the two Janus kinase 2 (Jak2) molecules, bound to the dimeric GHR, change, enabling Jak2 to crossphosphorylate itself and the tyrosine residues in the GHR cytosolic tails (4). The phosphorylation sites in GHR serve as docking sites for signalling and adapter molecules that transmit the GHR signals further into the cell and the nucleus (5).

GHR signalling has been shown to activate various signal transduction pathways, including the signal transducer and activator of transcription 5B (STAT5B) and the mitogen- activated protein kinase (MAPK) pathways (6). STAT proteins are recruited to phosphorylated tyrosine residues in receptor tails via their Src homology 2 (SH2) domains (7). Subsequently, STATs are phosphorylated by Jak2 followed by STAT dissociation from the receptor. Phosphorylated STATs dimerise, and the dimerised STATs translocate to the nucleus and activate their target genes (8). *In vivo* studies have identified STAT5B as the most important STAT for GHR signalling. Male STAT5B knock out mice were significantly smaller than wild type animals (9,10). Less important for GHR signalling is the MAPK pathway that has a general role in the regulation of cellular growth and differentiation (11). MAPK activation occurs via GH-induced phosphorylation of SH2 domain-containing transforming protein C (Shc), which results in initiation of activation of the MAPK cascade (12,13).

Many proteins have been identified to negatively regulate GHR signalling, including tyrosine phosphates and suppressors of cytokine signalling (SOCS). Several tyrosine phosphatases have been identified to dephosphorylate Jak2 and GHR, including SH2 domain-containing protein tyrosine phosphatases (SHP) 1 (14,15) and 2 (16-19) and PTP-H1 (20,21). GH induces expression of SOCS1, 2, 3 and cytokine-inducible SH2 protein (CIS). Overexpression of SOCS1 and 3 results in complete inhibition of GHR signalling, whereas SOCS2 and CIS overexpression affect GHR signalling only partially (22-26).

Like prolactin receptor (PrlR), erythropoietin receptor (EpoR), thrombopoetin receptor (TpoR), several interleukin and leptin receptors, the GHR is a member of the type I cytokine receptor superfamily (27). These receptors share two conserved regions, referred to as box-1 and box-2 (5). Both are essential for Jak2 binding and receptor signalling (28-30), whereas box-2, in addition, harbours the ubiquitin-dependent endocytosis (UbE) motif and is required for GHR endocytosis (31,32). The cytosolic tail of rabbit GHR contains nine tyrosine residues that can potentially be phosphorylated (33). Several studies have addressed the role of individual tyrosine residues in GHR signalling, of which 7 are conserved between human, rabbit and mouse GHR (Fig. 1A and B). The membrane-proximal tyrosine residues 332 and 337, as well as residues 487, 534, 566 and 627 were found to be phosphorylated in response to GH (34,35). Two studies identified tyrosine residues 534, 566 and 627 as recruitment sites for STAT5B and did not find a role for tyrosine residue 487 (36,37). An *in vivo* study showed that deletion of tyrosine residues 539, 545, 577, 606 and 639 in mouse GHR (534, 566, 595 and 627 in rabbit and

human GHR) resulted in a 70% decrease in STAT5B signalling, whereas a mouse GHR truncation containing only tyrosine residues 341 and 346 (332 and 337 in rabbit and human GHR) did not show any STAT5B activation (38). Although it is generally accepted that STAT5B signalling mainly depends on the distal tyrosine residues, it is still debated to which extent the individual tyrosine residues contribute to GHR, STAT5B and MAPK phosphorylation. Especially tyrosine residues 390, 436 and 487 need closer examination, since the i*n vivo* study by Rowland *et al* shows that STAT5B signalling depend on one or more of these residues (38).
In this study, we used biotinylated GH to activate, pull down and analyse GHR phosphorylation for a large set of GHR tyrosine mutants. In addition, we assayed the ability of the GHR mutants to activate STAT5B and MAPK. We find that tyrosine residues 487, 534 and 627 are the most important for GHR and STAT5B phosphorylation. In addition, we show that mutation of tyrosine residue 609 in GHR results in constitutive hyperphosphorylation of GHR, Jak2 and MAPK.

Experimental procedures

Cell culture

HEK293 cells were cultured in Dulbecco's modified eagle medium (DMEM), containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells were grown in culture medium without antibiotics. In experiments, in which cells were stimulated with biotinylated GH, we serum-starved them for two hours with DMEM with 4.5 g/l glucose.

DNA constructs, mutagenesis, transfections

Rabbit GHR in pcDNA3,1 was described before (39). We constructed the GHR tyrosine mutants by the introduction of tyrosine to phenylalanine mutations using the Quickchange mutagenesis kit from Stratagene. We used one primer set for tyrosine 332 and 337 (forward: GCCATTCAAGATAGCTTTAAGCCTGAATTCTTCAATGATGACTC, reverse:GAGTCATCATTGAAGAATTCAGGCTTAAAGCTATCTTGAATGGC). The other tyrosine residues were replaced using one primer set per tyrosine: 390 (forward: GGACGAACCAGCTGTTTCGAACCTGACATCCTG, reverse :CAGGATGTCAGGTTCGAAACAGCTGGTTCGTCC),436 (forward: CAA AATAACTCACCTTTCCATGATGTTTCTCCTGCTGCTCAG,reverse: CTGAGCAGCAGGAGAAACATCATGGAAAGGTGAGTTATTTTG), 487 (fo rward:CTGGCAAACATCGACTTTTTCGCCCAGGTTAGTGAC, reverse :GTCACTAACCTGGGCGAAAAAGTCGATGTTTGCCAG), 534 (forward: CATCATGGACAACGCCTTCTTCTGTGAAGCAGATGCC, reverse:GGCATCTGCTT CACAGAAGAAGGCGTTGTCCATGAT), 566 (forward:CCAGGAGGACATTTTCAT CACCACAGAAAGCC, reverse: GGCTTTCTGTGGTGATGAAAATGTCCTCCTGG, 595 (forward:GATGCCTGTCCCAGACTTTACCTCCATTCATTTAG, reverse: CTAAATGAATGGAGGTAAAGTCTGGGACAGGCATC) and 627 (forward: CTCTCATCGTGTGGCTTCGTGAGCACAGACCAAC, reverse GTTGGTCTGTGCTCACGAAGCCACACGATGAGAG). Flag-tagged wild type mouse Jak2 was a generous gift from Prof. Carter-Su. Transfections were done using FuGene6 (Roche), according to the manufacturer's instructions.

Materials and antibodies

Anti-GHR(T) and anti-GHR(B) were described before (39,40). Monoclonal anti-Flag (M2) was from Sigma and anti-STAT5B (C17) was from Santa Cruz Biotechnologies Inc. Monoclonal 4G10 anti-pY was obtained from Upstate (Millipore). Human GH was kindly provided by Eli Lilly & Co. Research labs (Indianapolis, IN). Biotinylated GH was made according to the manufacturer's instructions (Pierce, Rockford, IL). Protein A agarose beads were from Repligen Co. and Immunopure immnobilised streptavidin beads were purchased from Pierce.

Biotinylated-GH pull down and immunoprecipitation

Serumstarved HEK293 cells were stimulated with 180 ng/ml biotinylated GH for 15 minutes, washed once with cold PBS followed by a 15 minute lysis with cold lysis buffer (1% Trition-X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4 and 50 mM NaF in PBS). The cells were scraped, centrifuged to remove the nuclei and the post-nuclear supernatants were incubated for two hours with anti-GHR(T) and 45 minutes with protein A agarose beads (immunoprecpitation) or for one hour with streptavidin beads (biotinylated GHR pull down). The lysates, GHR immunoprecipitates and biotinylated GH-GHR complexes were subjected to SDS/PAGE.

Immunoblotting

After SDS-PAGE, proteins were transferred to an Immobilon-FL PVDF membrane (Millipore). The membranes were incubated with primary antibodies (as indicated) and secondary antibodies Alexa fluor 680 (Molecular Probes) or Alexa 800 IR-Dye (Rockland, Gilbertsville, PA) conjugated goat-anti-mouse or goat-anti-rabbit antibodies. Detection and quantification were performed with an Odyssey system (LI-COR Biosciences, Lincoln, NE).

Results

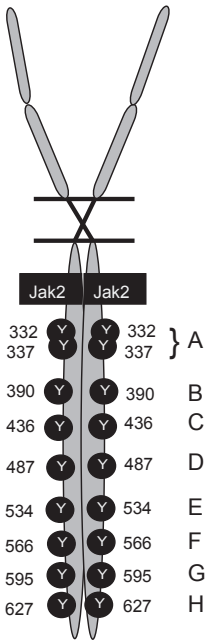
GHR tyrosine mutant screen

To identify tyrosine residues important for GHR phosphorylation and signalling, we constructed a large GHR tyrosine mutant library by mutagenesis of wild type rabbit GHR. The cytosolic tail of rabbit GHR contains nine tyrosine residues (Fig. 1A), of which seven are conserved among rabbit, human and mouse GHR (Fig. 1B). We assigned the tyrosine mutations A-H, as depicted in Fig. 1A and 1B.

HEK293 cells, expressing forty-seven different GHR tyrosine mutant constructs as well as wild type GHR were starved for two hours followed by a fifteen minute treatment with biotinylated GH. The GHRs were isolated with streptavidin beads and analysed for GHR phosphorylation. Both phosphorylated and total GHR levels were quantified and the ratios of phosphorylated GHR over total GHR were calculated (Fig. 2). The relative ratios varied between 0.010 for wild type GHR and 0.002 if all tyrosine residues were mutated (the background level). Importantly, the large window between positive (wild type GHR) and negative control (Yless) allows identification of GHR tyrosine mutant phenotypes.

The mutations in the membrane-proximal region had little effect on the pGHR/total GHR ratios. Interestingly, one mutant (Y627F) showed a twenty fold increase in ratio, which

A



B

	Mouse	Rabbit	Human
A {	341	332	332
	346	337	-
B	401	390	-
C	447	436	436
D	498	487	487
	539	-	-
E	545	534	534
F	577	566	566
G	606	595	595
H	639	627	627

Fig. 1. Tyrosine residues in the GHR
A. Schematic representation of rabbit GHR, in which the cytosolic tyrosine residues are indicated. B. Cytosolic tyrosine residues for mouse, rabbit and human GHR. In this paper, the tyrosine residues are referred to as characters A-H, as indicated in this table.

indicates that this GHR mutant is hyperphosphorylated. In addition to all the single mutations, Fig. 2A and B show the effects of various combinations of the membrane-proximal mutations. Mutations in the membrane-distal part of the GHR cytosolic tail are shown in fig. 2C: the latter affected GHR phosphorylation most severe.
In addition to GHR phosphorylation, phosphorylated STAT5B and MAPK were measured. Although we did measure several severe GHR phosphorylation phenotypes for the GHR mutants, we detected significant effects neither on STAT5 nor on MAPK phosphorylation (data not shown), except for GHR(Yless), suggesting a high level of redundancy for the tyrosine residues in the GHR cytosolic tail.
In summary, in accordance with data in literature, quantification of the pGHR/total GHR ratios of the GHR mutants showed that strong GHR phosphorylation phenotypes mainly result from mutation of the membrane-distal tyrosine residues.

Tyrosine residues 487 to 627 are important for GHR phosphorylation

Combinations with tyrosine residues 487 (D), 627 (H) and in particular 534 (E) resulted in considerable less GHR phosphorylation, while residues 566 and 595 have minor effects. To examine the role of distal tyrosine residues more precisely, we tested a set of multiple distal mutations, also in combination with membrane-proximal residues (A, A/C, A/C/D, A/C/D/E). Since tyrosine 390 (B) is absent in human GHR and did not contribute to GHR phosphorylation (Fig. 1B), it was not considered. Fig. 3 clearly shows that a gradual decrease in tyrosine residues results in a gradual decrease in GHR phosphorylation, confirming that the membrane-distal tyrosine residues are important for GHR phosphorylation. Whereas the results clearly show the effects of residues 487 (D)

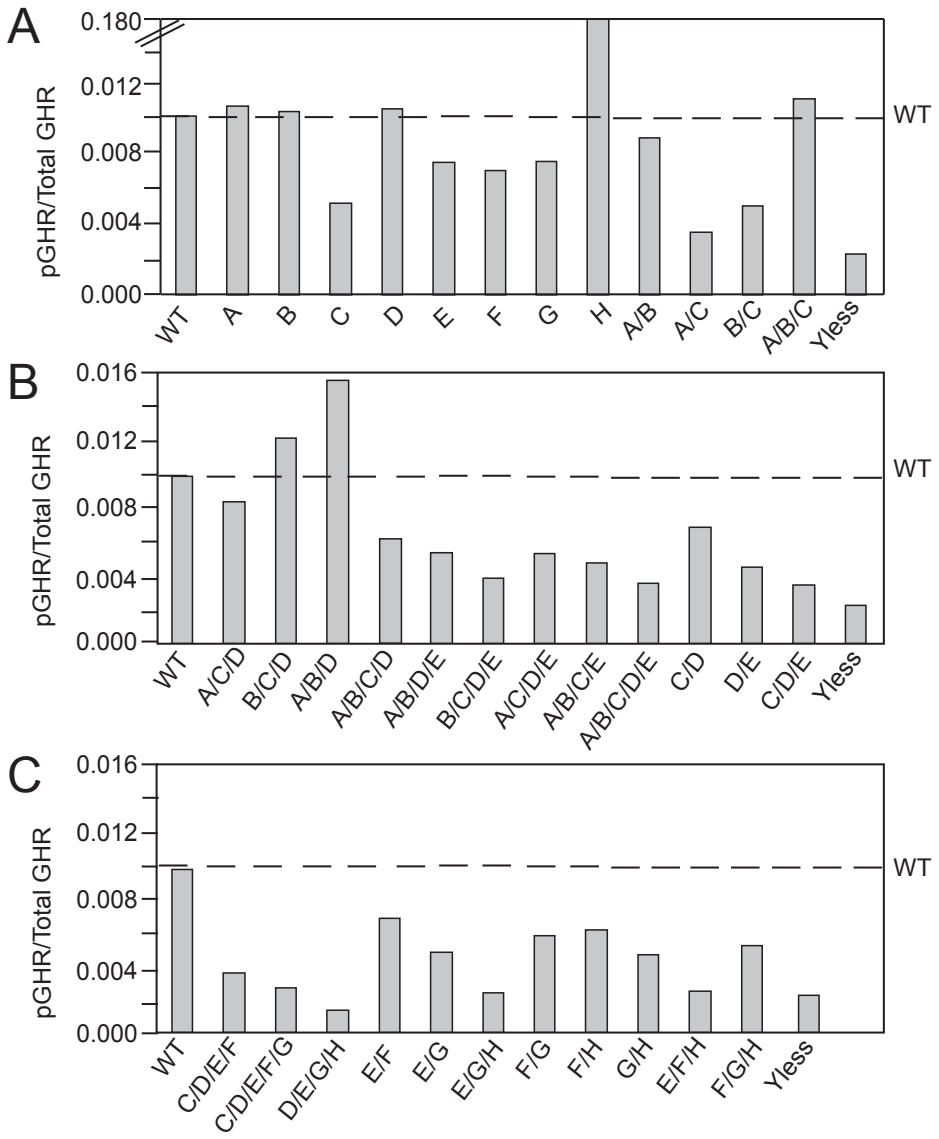


Fig. 2. The role of GHR cytosolic tyrosine residues in GHR phosphorylation
A.B.C. Wildtype and GHR Y-mutants, annotated according to Fig. 1, were expressed in cells. After starvation, biotinylated GH was allowed to bind GHR for 15 minutes at 37°C. GHR was pulled down from cell lysates and the samples were subjected to SDS-PAGE. After membrane transfer, phosphorylated and total GHR was detected and quantified followed by calculation of phosphorylated GHR divided by total GHR. The screen performed with all available GHR mutants was performed once.

and 534 (E), we were unable to determine whether tyrosine 566 (F), 595 (G) or 627 (H) or combinations result in the Yless GHR phenotype.

In addition to GHR phosphorylation, we measured STAT5B phosphorylation to determine whether the effect on GHR phosphorylation affected STAT5B activity (Fig. 3). Although Yless GHR abrogated STAT5B phosphorylation completely, the other GHR mutants did not decrease STAT5B phosphorylation much, suggesting that either tyrosine residues 566 (F), 595 (G) or 627 (H) are required for STAT5B activation.

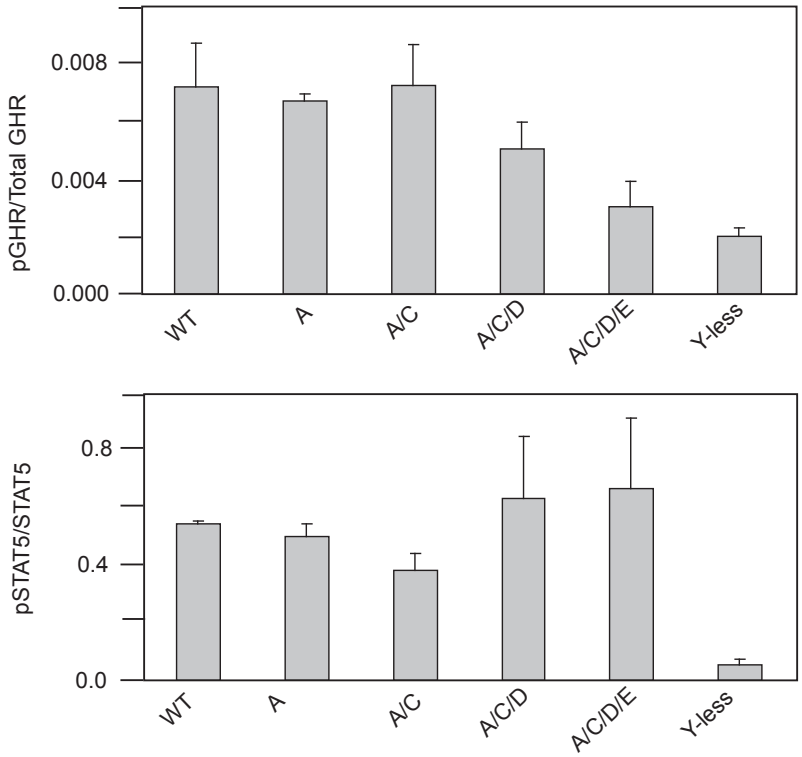


Fig. 3. Membrane-distal tyrosine residues are important for GHR phosphorylation
Wild type and GHR Y-mutants, annotated according to Fig. 1 were expressed in HEK293 cells. The cells were incubated with biotinylated GH and the GHR isolated and analysed as in Fig. 2. Phosphorylated and total GHR and phosphorylated and total STAT5B were detected and quantified. The ratios between phosphorylated GHR and total GHR (upper panel) and phosphorylated STAT5B and non-phosphorylated STAT5B (lower panel) are depicted. Both histograms represent means with standard errors of the mean of two independent experiments.

Tyrosine residues 487, 534 and 627 are important for STAT5B activation

In order to determine which tyrosine residues are important for STAT5B activation, we reconstituted the individual tyrosine residues in the Yless GHR. After GH treatment, we quantified the ratio of phosphorylated STAT5B over total STAT5B and found that tyrosine residues 487 (D), 534 (E) and 627 (H) were able to restore GHR activity towards STAT5B (Fig. 4). Reconstitution of the other tyrosines did not restore GHR activity. These data clearly demonstrate that GHR tyrosine residues Y487 (D), Y534 (E) and Y627 (H) are important for STAT5B activation by GHR.

GHR(Y627F) constitutively activates Jak2 and downstream effectors

The GHR pull down in Fig. 2 clearly demonstrates that the Y627 mutation results in an increase of GHR phosphorylation of more than twenty fold. Surprisingly, additional mutations in Y534, Y566 or Y595 resulted in loss of this phenotype. Complete sequencing of the GHR(Y627F) construct confirmed that the construct did not have additional mutations.

Fig. 5A shows the phenotypes of all the GHR mutants with a single tyrosine residue mutated: Y627F (H) clearly demonstrated the strongest phenotype. First, we tested whether this phenotype depended on the fifteen minute GH treatment (Fig. 5B).

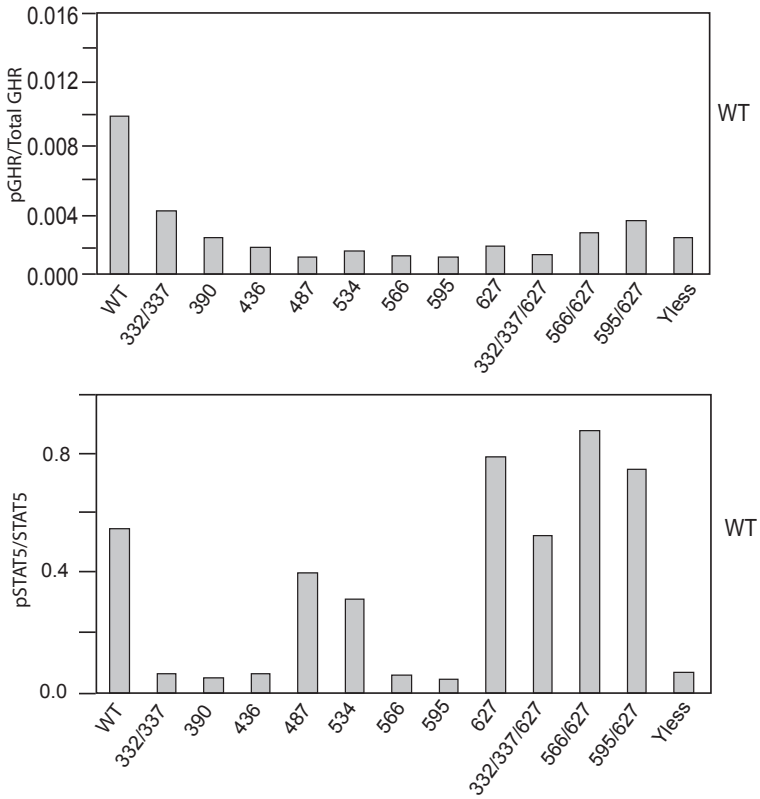


Fig. 4. Reconstitution of tyrosine residues 487, 534 and 627 restores GHR phosphorylation
GHRs, in which all tyrosine residues were mutated except for the indicated tyrosine residue, were expressed in cells. The experiment was performed as described in Fig. 3. Phosphorylated and total GHR (upper panel) and STAT5B (lower panel) were detected and quantified. The ratios are depicted in the figure.

We noticed a very strong pY signal in both non-treated and GH-treated cells, demonstrating that the hyperphosphorylation phenotype of GHR did not result from GH stimulation. STAT5B activation only occurred after GH stimulation, indicating that without GH the tyrosine residues that are involved in STAT5B activation (Y487, Y534) are not (hyper) phosphorylated. Apparently, only after GH stimulation, they become phosphorylated and/or available for STAT5B. Interestingly, we found that MAPK, but not STAT5 was hyperactivated (Fig. 5A), suggesting that not the membrane-distal tyrosine residues of the GHR, but rather the membrane-proximal tyrosines are hyperphosphorylated in GHR(Y627F). In addition to GHR and MAPK, also Jak2 was hyperphosphorylated. This is remarkable as it shows that (endogenous) Jak2, is present on the GHR in an active conformation: according to the current view, it means that the two Jak2s can already phosphorylate each other without a conformational change brought about by GH binding (3,41). This finding is in favour of the hypothesis that phosphatases play a major role as kinases regulators (42). Together, we showed that mutation of tyrosine residue 627 of GHR results in constitutive hyperphosphorylation of GHR, Jak2 and MAPK.

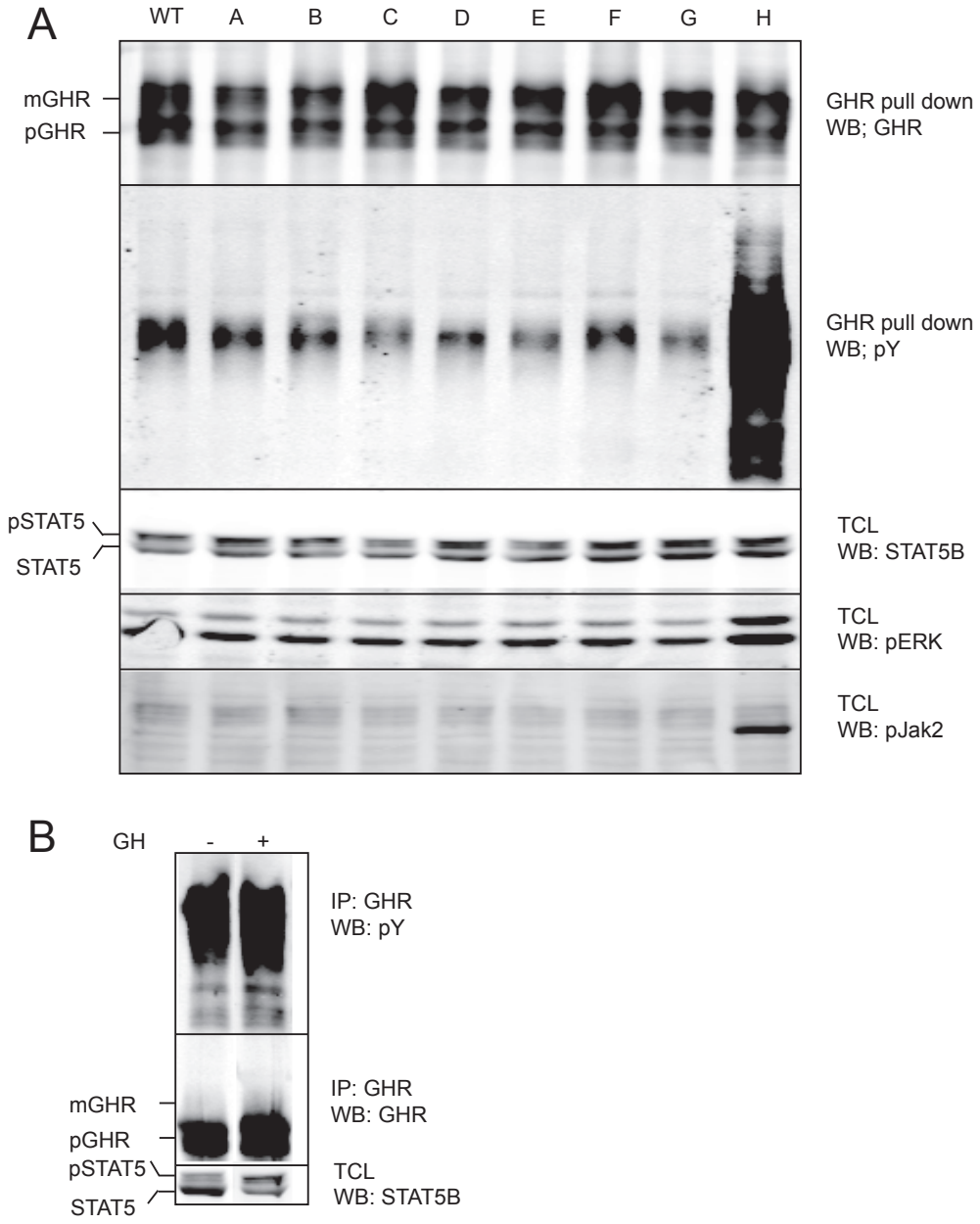


Fig. 5. Mutation of tyrosine residue 627 results in GHR, Jak2 and MAPK hyperphosphorylation
A. GHRs, mutated as indicated, were expressed in HEK293 cells. The characters indicate single Y mutations as in Fig. 1. The experiment was performed as in Fig. 3. After cell lysis, GHR pull down samples and total cell lysates (TCL) were subjected to SDS-PAGE. After membrane transfer, the western blots were detected for the indicated proteins. B. GHR mutant H was expressed in cells and treated without and with GH. Cell lysates and GHR-immunoprecipitates were subjected to SDS-PAGE and western blotting followed by detection for the indicated proteins. Data are representative of at least three experiments.

Discussion

In this study, we showed that membrane-distal tyrosine residues 487, 534 and 627 contribute to GHR phosphorylation. Moreover, GHR with only one tyrosine residue in the positions of either 487, 534 and 627 is sufficient to rescue STAT5B activation. Whereas tyrosine residues 534 and 627 are considered to be involved in STAT5B phosphorylation in most studies, the role of Y487 is still under debate. Furthermore, Y566 has been identified in several studies to be important for STAT5B signalling, but we find no clear role of this particular tyrosine residue in STAT5B activation. At least, this residue cannot act as an individual tyrosine residue like Y487, Y534 and Y627 to rescue STAT5B activation.

In a study describing knockin mice, expressing different mouse GHR truncations, with reduced postnatal growth and increased obesity, the phenotype of truncation 391, in which only mouse tyrosine residues 341 and 346 (rabbit 332 and 337) were present, was severe. Truncation 569, in which in addition to mouse tyrosine residues 341 and 346 (rabbit 332 and 337), mouse tyrosine residues 401, 447 and 498 (rabbit 390, 436 and 487) were intact, showed a milder phenotype. Interestingly, the authors identified a region in GHR, including mouse tyrosine residues 401, 447 and 498 (rabbit 390, 436 and 487), to be involved in the promotion of postnatal growth (38,43). Our current study suggests that, from these three tyrosine residues, only tyrosine residue 487 (mouse 498) in rabbit GHR is important for STAT5B activation.

Another study from Uyttendaelle *et al* showed by a mammalian protein-protein interaction trap (MAPPIT) that rabbit Y534, Y566 and Y627 are important for STAT5B binding and activation. We confirmed the role of Y534 and Y627, but we found Y487 to be important as well and we did not identify Y566 as an important residue for STAT5B activation. The reason for these different findings might be that Uyttendaelle *et al* mainly focussed on STAT5B binding and our study only measured STAT5B activation. Based on their observation that tyrosine residue 487 is not a STAT5B recruitment site, Uyttendaelle *et al* concluded that SOCS2, which binds via tyrosine residue 487, does not act by competing with STAT5B binding. Considering our data, we propose that SOCS2 inhibits GHR signalling by binding competition.

We observed a strong GHR phosphorylation phenotype if a single residue, Y627, was replaced by phenylalanine. In addition, Jak2 and MAPK were hyperphosphorylated. The observation that MAPK and not STAT5B phosphorylation was affected, suggests that the membrane-proximal rather than the membrane-distal tyrosine residues are hyperphosphorylated, since MAPK activation has been shown to primarily depend on the proximal Jak2 activation domain. It is difficult to understand how the Y627F mutation gives rise to the observed phenotype, especially since the addition of another tyrosine residue results in loss of the phenotype. A possible scenario is that Y627 is part of a phosphatase binding site. Alternatively, Y627 might be required for Jak2 inactivation/degradation, or Y627 could be essential for Jak2 dissociation from the GHR. However, the finding is potentially very interesting. Uncontrolled MAPK signalling is often associated with a variety of cancers (12), suggesting that GHR, Jak2 and MAPK hyperphosphorylation by the mutation of Y627 in GHR could result in tumour development.

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References

1. Ayuk, J., and Sheppard, M. C. (2006) *Postgrad Med J* **82**, 24-30
2. Moller, N., Vendelbo, M. H., Kampmann, U., Christensen, B., Madsen, M., Norrelund, H., and Jorgensen, J. O. (2009) *Clin Nutr* **28**, 597-603
3. Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) *Nat Struct Mol Biol* **12**, 814-821
4. Waters, M. J., Hoang, H. N., Fairlie, D. P., Pelekanos, R. A., and Brown, R. J. (2006) *J Mol Endocrinol* **36**, 1-7
5. Postel-Vinay, M. C., and Finidori, J. (1995) *Eur J Endocrinol* **133**, 654-659
6. Rosenfeld, R. G., Belgorosky, A., Camacho-Hubner, C., Savage, M. O., Wit, J. M., and Hwa, V. (2007) *Trends Endocrinol Metab* **18**, 134-141
7. O'Sullivan, L. A., Liongue, C., Lewis, R. S., Stephenson, S. E., and Ward, A. C. (2007) *Mol Immunol* **44**, 2497-2506
8. Okada, S., and Kopchick, J. J. (2001) *Trends Mol Med* **7**, 126-132.
9. Teglund, S., McKay, C., Schuetz, E., van Deursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93**, 841-850
10. Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S. H., Ram, P. A., Waxman, D. J., and Davey, H. W. (1997) *Proc Natl Acad Sci U S A* **94**, 7239-7244.
11. Cobb, M. H., and Goldsmith, E. J. (1995) *J Biol Chem* **270**, 14843-14846
12. Zhang, W., and Liu, H. T. (2002) *Cell Res* **12**, 9-18
13. VanderKuur, J. A., Butch, E. R., Waters, S. B., Pessin, J. E., Guan, K. L., and CarterSu, C. (1997) *Endocrinology* **138**, 4301-4307.
14. Jiao, H., Berrada, Yang, W., Tabrizi, M., Platanias, L. C., and Yi, T. (1996) *Mol Cell Biol* **16**, 6985-6992
15. Hackett, R. H., Wang, Y. D., Sweitzer, S., Feldman, G., Wood, W. I., and Larner, A. C. (1997) *J. Biol. Chem.* **272**, 11128-11132
16. Stofega, M. R., Herrington, J., Billestrup, N., and Carter-Su, C. (2000) *Mol Endocrinol* **14**, 1338-1350
17. Stofega, M. R., Wang, H., Ullrich, A., and Carter-Su, C. (1998) *J Biol Chem* **273**, 7112-7117
18. Ohnishi, H., Kubota, M., Ohtake, A., Sato, K., and Sano, S. (1996) *J Biol Chem* **271**, 25569-25574
19. Stofega, M. R., Argetsinger, L. S., Wang, H., Ullrich, A., and Carter-Su, C. (2000) *J Biol Chem* **275**, 28222-28229
20. Pasquali, C., Curchod, M. L., Walchli, S., Espanel, X., Guerrier, M., Arigoni, F., Strous, G., and Hooft Van Huijsduijnen, R. (2003) *Mol Endocrinol* **17**, 2228-2239
21. Pilecka, I., Whatmore, A., Hooft van Huijsduijnen, R., Destenaves, B., and Clayton, P. (2007) *Trends Endocrinol Metab* **18**, 12-18
22. Adams, T. E., Hansen, J. A., Starr, R., Nicola, N. A., Hilton, D. J., and Billestrup, N. (1998) *J. Biol. Chem.* **273**, 1285-1287
23. Paul, C., Seiliez, I., Thissen, J. P., and Le Cam, A. (2000) *Eur J Biochem* **267**, 5849-5857
24. Tollet-Egnell, P., Floresmorales, A., Stavreusevers, A., Sahlin, L., and Norstedt, G. (1999) *Endocrinology* **140**, 3693-3704
25. Hansen, J. A., Lindberg, K., Hilton, D. J., Nielsen, J. H., and Billestrup, N. (1999) *Mol. Endocrinol.* **13**, 1832-1843
26. Ram, P. A., and Waxman, D. J. (1999) *J. Biol. Chem.* **274**, 35553-35561
27. Grotzinger, J. (2002) *Biochim Biophys Acta* **1592**, 215-223
28. Frank, S. J., Gilliland, G., Kraft, A. S., and Arnold, C. S. (1994) *Endocrinology* **135**, 2228-2239
29. Sotiropoulos, A., Perrot Applanat, M., Dinerstein, H., Pallier, A., Postel Vinay, M. C., Finidori, J., and Kelly, P. A. (1994) *Endocrinology* **135**, 1292-1298
30. Pelletier, S., Gingras, S., Funakoshi-Tago, M., Howell, S., and Ihle, J. N. (2006) *Mol Cell Biol* **26**, 8527-8538
31. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., and Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11349-11353
34. VanderKuur, J. A., Wang, X. Y., Zhang, L. Y., Allevato, G., Billestrup, N., and CarterSu, C. (1995) *J. Biol. Chem.* **270,**, 21738-21744.

35. Wang, X. Z., Darus, C. J., Xu, B. X. C., and Kopchick, J. J. (1996) *Mol Endocrinol* **10,**, 1249-1260.
36. Uyttendaele, I., Lemmens, I., Verhee, A., De Smet, A. S., Vandekerckhove, J., Lavens, D., Peelman, F., and Tavernier, J. (2007) *Mol Endocrinol* **21**, 2821-2831
37. Hansen, L. H., Wang, X. Z., Kopchick, J. J., Bouchelouche, P., Nielsen, J. H., and Billestrup, N. (1996) *J. Biol. Chem.* **271**, 12669-12673
38. Rowland, J. E., Lichanska, A. M., Kerr, L. M., White, M., d'Aniello, E. M., Maher, S. L., Brown, R., Teasdale, R. D., Noakes, P. G., and Waters, M. J. (2005) *Mol Cell Biol* **25**, 66-77
39. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) *EMBO J.* **15**, 3806-3812
40. van Kerkhof, P., Govers, R., Alves dosSantos, C. M. A., and Strous, G. J. (2000) *J Biol Chem* **275**, 1575-1580
41. Gent, J., van Kerkhof, P., Roza, M., Bu, G., and Strous, G. J. (2002) *Proc Nat Acad Sci USA* **99**, 9858-9863
42. Soulsby, M., and Bennett, A. M. (2009) *Physiology (Bethesda)* **24**, 281-289
43. Schirra, H. J., Anderson, C. G., Wilson, W. J., Kerr, L., Craik, D. J., Waters, M. J., and Lichanska, A. M. (2008) *PLoS One* **3**, e2764

CHAPTER VI

Summarising Discussion

Joyce Putters

Department of Cell Biology and Institute of Biomembranes
University Medical Center Utrecht

Summarising discussion

This thesis contains four experimental chapters, which describe the role of phosphorylation and ubiquitination events in GHR trafficking and signalling. Chapters II and III describe the mechanisms how βTrCP and Jak2 regulate GHR endocytosis. In chapter IV, endogenous β-TrCP isoforms and splice variants were characterised. Finally, in chapter V, we report the identification of GHR tyrosine residues that are important for GHR phosphorylation and signal transduction. In this summarising discussion, a model is proposed and discussed based on the findings described in this thesis and on previous results.

GHR endocytosis and signalling

GH has an important role in postnatal longitudinal growth, but is also involved in protein, carbohydrate and fat metabolism. Some mutations in the GHR gene result in the Laron or growth hormone insensitivity syndrome (GHIS). They were found predominantly in the GHR extracellular domain disabling GHR-GH binding or in the GHR cytosolic tail abrogating STAT5B activation. GHIS is characterised by a severe IGF-I deficiency and results in dwarfism (1). Muscle wasting conditions can be symptoms of underlying diseases such as cancer and AIDS, but also in bedridden conditions and severe burning. Muscle wasting has been associated with high levels of circulating GH and low IGF-I levels (2,3). In wasting conditions, it has been suggested that this is a consequence of the upregulation of the ubiquitin system, resulting in GHR downregulation (4). Altogether, GHR expression at the cell surface is essential for promoting growth, but also for proper metabolism in the cell (5).

Functional GHR expression at the cell surface is determined by several processes, including GHR synthesis, GHR shedding and GHR endocytosis. Although ongoing GHR synthesis is an important determinant of the number of GHRs at the cell surface, it is a difficult parameter to manipulate. Since GHR shedding affects about 20% of the GHRs and GHR endocytosis regulates about 80% of the GHRs at the cell surface (6), inhibition of GHR endocytosis could be a means to prevent muscle wasting in patients. An important part of this thesis describes the investigation of the mechanism of GHR endocytosis, with the focus on the role of βTrCP and Jak2 in this process.

Besides the regulation of GHR availability at the cell surface, it is important to know how the GHR signal transduction pathways are activated to exert the biological effects of GH. Several studies have investigated which of the tyrosines of GHR are essential for initiating GHR signal transduction, but it is still under debate how the different tyrosine residues exactly contribute to GHR, STAT5B and MAPK phosphorylation. Therefore, we investigated the role of the nine rabbit cytosolic GHR tyrosines in GHR signalling.

Model of GHR endocytosis

Previously, data from our group revealed that GHR endocytosis is dependent on an intact ubiquitin proteasome system (7) and on the UbE-motif (8). We describe in chapter II that the F-box protein βTrCP, as part of a SCF complex, is essential for GHR endocytosis via binding to the GHR UbE motif. Moreover, βTrCP is involved in K48-linked polyubiquitination of the GHR, either directly or indirectly (Chapter III). Interestingly, βTrCP isoform 2 rather than 1 is essential for GHR degradation. Due to its exclusive location in the cytosol, the splice variant βTrCP2γ is likely the real actor in the endocytic process (Chapter IV).

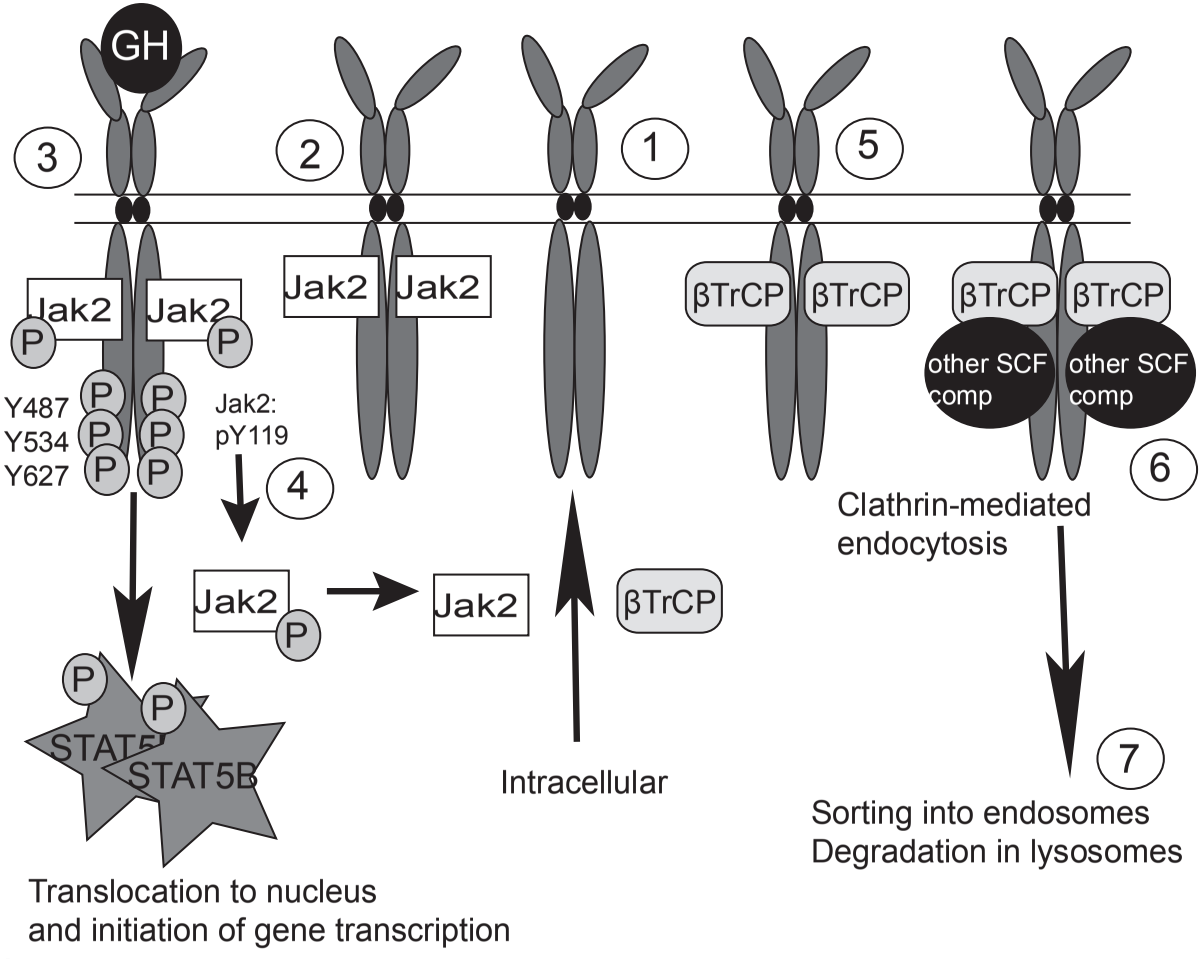

Figure 1: Model of GHR endocytosis and signalling

Upon arrival at the cell surface, binding of Jak2 or βTrCP to the GHR determines whether the GHR is able to initiate signal transduction or whether the GHR is endocytosed via SCFβTrCP and eventually degraded in the lysosome. For a detailed explanation of events, see text.

Another essential player in GHR endocytosis is the kinase Jak2, which inhibits GHR endocytosis and degradation independently of its kinase activity (Chapter III). Jak2 binding prevents selection of the GHR for endocytosis by the $SCF^{\beta TrCP}$ complex, while Jak2 dissociation from the GHR by phosphorylation enables cargo selection of the GHR by $SCF^{\beta TrCP}$. Although GH-induced and constitutive GHR endocytosis were previously proposed to be two different pathways, we found that both endocytosis *modi* depend on the ubiquitin proteasome system, clathrin, βTrCP, and the UbE motif in the GHR. In contrast to other reports on cytokine receptors, the DSGXXS motif and phosphorylated tyrosine residues in the GHR cytosolic tail are not required.
In chapter V, we identified tyrosines 487, 534 and 627 in the GHR to be important for STAT5B signalling. In addition, we report that mutation of tyrosine residue 627 in GHR results in hyperphosphorylation of GHR, Jak2 and MAPK, but not STAT5B.
The findings, described in chapters II, III and IV, contribute to a better understanding of GHR endocytosis and chapter V identifies important residues for GHR signalling. In Fig. 1, I summarised our current knowledge in a model that describes the molecular details of GHR endocytosis and signalling.
A GHR dimer, which is preformed in the ER, arrives at the cell surface, where it can bind either βTrCP or Jak2, depending on the relative concentration of these two proteins (1). Upon Jak2 binding, the GHR is kept at the cell surface (2) where it is able to bind GH and to activate Jak2, followed by Jak2-induced phosphorylation of Jak2 itself and of the GHR. Tyrosine residues 487, 534 and 627 in the GHR cytosolic tail are the most important residues for STAT5B activation, which is responsible for most of the effects of GH signalling (3). After GH stimulation, phosphorylation of tyrosine 119 in Jak2 results in dissociation of phosphorylated Jak2 from the GHR (4). Subsequently, the GHR can bind Jak2 again and initiate signal transduction or the GHR binds βTrCP (5) and is selected for clathrin-mediated endocytosis, followed by $SCF^{\beta TrCP}$ assembly (6) and GHR sorting and degradation in the endosomal-lysosomal system (7).

The molecular mechanism of the model

Although we filled several important gaps in understanding GHR endocytosis, some questions remain. A complicating factor in deciphering the mechanism of ubiquitin-dependent GHR endocytosis is the fact that GHR ubiquitination is not required for GHR endocytosis (9). Since it is known that one or more active E3 ligases, at least βTrCP, are required for GHR endocytosis, there are several possible scenarios. It might be that the ubiquitinated substrate (or factor X) that is crucial for GHR endocytosis is a GHR-associated protein. In fact, βTrCP itself might be factor X. If GHR endocytosis requires another factor to be ubiquitinated (by βTrCP or another E3 ligase), it might be that this factor is degraded by the proteasome. How such a proteasomal action can induce endocytosis remains unclear. Another scenario could be that the ubiquitinated factor X recruits an endocytic adapter like EPS15 or epsin, which binds clathrin and specifically facilitates GHR endocytosis.
As it is generally accepted that cargo selection at the multivesicular bodies (MVBs) is ubiquitination-driven, it might be that transport through MVB depends on ubiquitination as well. Data from our lab indicate that GHR truncation 349 indeed requires an intact UbE-motif in order to be sorted in the MVB. Although speculative, it could be that GHR needs to be ubiquitinated in order to be efficiently recognised by the ESCRT system, as

it has been reported for receptor tyrosine kinases. As a lysine-less GHR is degraded in lysosomes, GHR ubiquitination might also be a bystander effect. This, again, points to the involvement of factor X, but now at the level of the MVBs.

In chapters II and IV, we describe that βTrCP binds concentration-dependent via the UbE-motif to the GHR without any post-translational modifications. Since also Jak2 binds GHR without requirements for GHR modifications and box-1 and UbE motifs in GHR are located close to each other, it is tempting to speculate that βTrCP and Jak2 bind competitively. Since productive binding of Jak2 with cytokine receptors involves multiple interactions with several amino acids including box-1 and box-2, it is expected that Jak2 binding prevents βTrCP interaction. Data from Pelletier *et al* (10) indeed show that Jak2 binding might cover a region which completely overlaps with the UbE motif. A functional consequence of concentration-dependent binding of βTrCP to the GHR is that high GHR levels bind free βTrCP, which could affect the other cellular functions of βTrCP. βTrCP substrate binding affinities will then determine which substrate is 'treated' first. As a Jak2-GHR interaction is only signalling-competent if both GHR tails possess a Jak2 molecule, the model still lacks several details. It is well possible that one Jak2, although signalling-incompetent, is able to block endocytosis via βTrCP, considering that also βTrCP likely functions as a dimer via its dimerisation domain.

In chapter III, we showed that Jak2 activation induces its own phosphorylation and dissociation from the GHR allowing βTrCP to start GHR degradation. However, it is likely that cells have other mechanisms to interrupt the GHR-Jak2 interaction in order to decrease the number of GHRs at the cell surface. One example might be a protein that interferes with the GHR-Jak2 interaction, although no obvious candidates are known at the moment. Another possibility could be that stress-induced Jak2 degradation is a means of GHR downregulation. Preliminary data from our lab support this mechanism of regulating the number of Jak2 molecules by degradation (Nespital and Strous, unpublished).

Both βTrCP and Jak2 have been associated with the trafficking of other cytokine receptors. βTrCP stimulates IFNAR1 subunit of the type I interferon (11), prolactin (12) and Epo receptor (13) endocytosis and degradation via the DSGXXS-motif. In contrast, βTrCP induces GHR degradation by binding to the UbE-motif, despite the presence of a DSGXXS motif in the GHR cytosolic tail. Whereas the role of βTrCP is limited to cytokine receptor degradation, the role of Jak2 is both on the trafficking and signalling and varies among the type I cytokine receptors. Jak2 binds the immature EpoR and increases EpoR cell surface expression by stimulating EpoR folding and Golgi processing. Jak2 and Tyk2 promote TpoR cell surface expression by stimulating recycling and enhancing protein stability, while internalisation is not affected. This is in contrast to the GHR, for which we show in chapter III that Jak2 severely inhibits GHR endocytosis. The different mechanisms of action of βTrCP and Jak2 on different cytokine receptors suggest that the intracellular tails of cytokine receptor constitute unique features. For example, the use of a different motif by βTrCP to bind GHR compared to other cytokine receptors allows the GHR to endocytose ligand-independent, since the DSGXXS-motif requires serine phosphorylation and the UbE-motif does not. Jak2 binding to GHR inhibits constitutive GHR endocytosis, whereas Jak2 does not affect internalisation of cytokine receptors that endocytose exclusively ligand-dependent. Whether βTrCP and Jak2 are involved in the trafficking of all cytokine receptors remains to be investigated. In the meantime, the role of the DSGXXS motif in the

GHR, conserved from zebra fish to human, continues to be a mystery. Apparently, every cytokine receptor orchestrates its own degradation in a unique way.

Interestingly, we found that a single mutation of tyrosine residue 627 (Y627F) results in constitutive hyperphosphorylation of GHR, Jak2 and MAPK, but not of STAT5B. Since MAPK, but not STAT5 was hyperphosphorylated, it is possible that the membrane-proximal GHR tyrosines are hyperphosphorylated. However, this does not explain why the hyperphosphorylation is not observed upon additional removal of tyrosine residues 534, 566 and 595. Most probable scenario is that tyrosine 627 is responsible for recruiting a protein tyrosine phosphatase. To elucidate this further, more experiments need to be done.

Physiological consequences of the model of GHR endocytosis

Obviously, a receptor with such an impact on major cellular functions must be tightly regulated. One striking example of the importance of the regulation of cell surface GHR for the signalling outcome is sexual dimorphism in the liver. Male GH secretion is mainly pulsatile, while female GH secretion is more continuous. This difference in GH secretion results in the activation of different genes of the cytochrome P (CYP) family in the liver (14), of which many are involved in the metabolism of steroids and fatty acids.

Cells with many GHRs in complex with Jak2 will be rapidly downregulated upon GH-stimulation. If these cells have a limited capacity to synthesize new GHRs (low mRNA numbers) these cells will respond to GH spikes, but will become GH-insensitive for a relative long period. Cells with a high biosynthetic potential (e.g. hepatocytes) will respond to GH-spikes, but will remain GH-sensitive after GH-stimulation. The cellular level of βTrCP2 will determine the rate of GHR internalisation, indicating that both Jak2 and βTrCP2 are important for the sex-dependent differences in gene expression in the liver.

Measurement of RNA- and protein levels of Jak2 and βTrCP2 revealed that relatively high Jak2 levels correlate with high GHR protein levels. These data suggest that a high Jak2 level compared to βTrCP2 increases GH-sensitivity, whereas cells in which the situation is reversed are relatively GH-insensitive. Together with the GHR synthetic capacity, this mechanism provides for a subtle GH-sensitivity regulation system. It is likely that the concentration of Jak2 substrates also plays an important role. A high concentration of other Jak2 substrates in the cell results in lower numbers of Jak2 available to bind to the GHR and thereby in a lower number of GHRs at the cell surface.

As described above, muscle wasting conditions result in upregulation of components of the ubiquitin system. Thus, it could be that βTrCP is such a component. In addition to low GHR cell surface expression, high glucocorticoid levels have been associated with muscle wasting as well. Moreover, glucocorticoids stimulate transcription of components of the ubiquitin system. Upregulation of βTrCP by glucocorticoids might be a mechanism to downregulate functional GHR cell surface expression in wasting conditions.

Increased GHR signalling results in elevated IGF-I levels, which have been associated with a number of cancers (15). Blocking GHR-activation results in a 80% decrease of IGF-I levels, indicating that reduced GHR signalling can prevent tumour development. This is exemplified in Laron diseased people who do not suffer from any cancer as well as in tall women who have a 50% higher risk of breast cancer (16-18). Considering this, high βTrCP2

levels, as well as low Jak2 levels, would be inhibiting tumour development, whereas high non-functional βTrCP (for example isoform 1 or non-functional splice variants) or high Jak2 levels would be tumour-promoting.

Biological actions of GH in the immune system include the enhancement of T- and B-cell development, stimulation of antibody production and anti-apoptotic effects in immune cells. B-cells are probably the major site of action of GH in the immune system, since the GHR is prominently expressed in B-cells. GH enhances immunoglobulin synthesis, both IGF-I-dependent and independent, indicating that the regulation of the number of GHRs at the cell surface of B-cells is important. Anti-apoptotic effects were observed in immune cells that overexpressed GH, indicating that increased GHR signalling can result in tumour development in immune cells, again suggesting that the regulation of GHRs at the cell surface is of major importance (19).

The findings of this thesis have resulted in a substantially increased understanding of the regulation of GHR cell surface expression and signalling. This knowledge can be used for the development of treatments against GHR-related disorders, like cancer, GHIS and wasting conditions.

References

1. Savage, M. O., Burren, C. P., Blair, J. C., Woods, K. A., Metherell, L., Clark, A. J., and Camacho-Hubner, C. (2001) *Horm Res* **55 Suppl 2**, 32-35
2. Bing, C. (2005) *Br J Nutr* **93**, 761-763
3. Tisdale, M. J. (2001) *Nutrition* **17**, 438-442.
4. Lin, S. Y., Chen, W. Y., Lee, F. Y., Huang, C. J., and Sheu, W. H. (2004) *Am J Physiol Endocrinol Metab* **288**, E493-501
5. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinology* **143**, 1243-1252
6. van Kerkhof, P., and Strous, G. J. (2001) *Biochem Soc Trans* **29**, 488-493.
7. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) *EMBO J.* **15**, 3806-3812
8. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) *EMBO J.* **18**, 28-36.
9. van Kerkhof, P., Govers, R., Alves dosSantos, C. M. A., and Strous, G. J. (2000) *J Biol Chem* **275**, 1575-1580
10. Pelletier, S., Gingras, S., Funakoshi-Tago, M., Howell, S., and Ihle, J. N. (2006) *Mol Cell Biol* **26**, 8527-8538
11. Kumar, K. G., Tang, W., Ravindranath, A. K., Clark, W. A., Croze, E., and Fuchs, S. Y. (2003) *Embo J* **22**, 5480-5490
12. Li, Y., Suresh Kumar, K. G., Tang, W., Spiegelman, V. S., and Fuchs, S. Y. (2004) *Mol. Cell. Biol.* **24**, 4038-4048
13. Meyer, L., Deau, B., Forejtnikova, H., Dumenil, D., Margottin-Goguet, F., Lacombe, C., Mayeux, P., and Verdier, F. (2007) *Blood* **109**, 5215-5222
14. Lichanska, A. M., and Waters, M. J. (2008) *Trends Genet* **24**, 41-47
15. Khandwala, H. M., McCutcheon, I. E., Flyvbjerg, A., and Friend, K. E. (2000) *Endocr Rev* **21**, 215-244
16. Shevah, O., and Laron, Z. (2007) *Growth Horm IGF Res* **17**, 54-57
17. Gunnell, D., Okasha, M., Smith, G. D., Oliver, S. E., Sandhu, J., and Holly, J. M. (2001) *Epidemiol Rev* **23**, 313-342
18. Ahlgren, M., Melbye, M., Wohlfahrt, J., and Sorensen, T. I. (2004) *N Engl J Med* **351**, 1619-1626
19. Hattori, N. (2009) *Growth Horm IGF Res* **19**, 187-197

Nederlandse samenvatting

Het groeien van het menselijk lichaam tot een volwassen persoon is een complex proces, dat door vele verschillende factoren gereguleerd wordt. Een belangrijke factor is groeihormoon, een eiwit dat gemaakt wordt in de cellen van de hypofyse-voorkwab van de hersenen en wordt uitgescheiden naar de bloedbaan. Gedurende de jeugd is groeihormoon in belangrijke mate bepalend voor de lengtegroei. Te weinig groeihormoon leidt dan ook tot dwerggroei en teveel tot reuzengroei. Na de puberteit veroorzaakt teveel groeihormoon vergroeiingen van gezicht, handen en voeten. Groeihormoon is niet alleen belangrijk voor lengtegroei, het zorgt ook voor de aanmaak en instandhouding van spierweefsel en voor een optimaal eiwit-, vet- en suikermetabolisme bij zowel kinderen als volwassenen. Ook is groeihormoon betrokken bij kanker, diabetes en de vermagering van ernstig zieke patiënten.

De effecten van groeihormoon komen alleen tot stand wanneer groeihormoon in staat is een signaal te geven aan cellen die het werk moeten doen. Dit gebeurt via de groeihormoonreceptor op het oppervlak van cellen. De groeihormoonreceptor is een eiwit dat in het celmembraan (de buitenkant van de cel) verankerd zit, met een extracellulair deel buiten en een intracellulair deel binnen in de cel. Groeihormoon, dat in de bloedbaan rondzwerft, bindt aan het extracellulaire deel van de groeihormoonreceptor. Eén groeihormoonmolecuul bindt aan twee groeihormoonreceptor moleculen, een groeihormoonreceptor-*dimeer*. Deze binding activeert het enzymeiwit Jak2, waarvan er twee aan het intracellulaire deel van de groeihormoonreceptor dimeer vast zitten. Jak2, een kinase dat drie keer zo groot is als het intracellulaire gedeelte van de groeihormoonreceptor, zet fosfaatgroepen op tyrosine residuen van zichzelf en de groeihormoonreceptor. Dit leidt weer tot binding en activatering van andere signaaltransductie-eiwitten, zoals STAT5B, MAPK en IP3 kinase eiwitten. Activatie van deze eiwitten resulteert in de eigenlijke respons van de cel op groeihormoon: (spier)eiwitsynthese, vet afbraak, celdeling. Dit laatste proces wordt vooral gecontroleerd door het hormoon IGF1 dat op commando van groeihormoon vooral door de lever wordt gemaakt. Aangezien groeihormoon alleen werkt als het aan de groeihormoonreceptor bindt, is de hoeveelheid groeihormoonreceptoren op het celopppervlak van cellen cruciaal. Deze hoeveelheid wordt in belangrijke mate bepaald door de snelheid waarmee de groeihormoonreceptor wordt afgebroken.

In dit proefschrift hebben we onderzoek gedaan naar de afbraak van de groeihormoonreceptor en de manier waarop deze receptor de cel aanzet tot een specifieke respons. Na de synthese van de groeihormoonreceptor en zijn transport naar het celoppervlak, wordt de groeihormoonreceptor, onafhankelijk van groeihormoonbinding, opgenomen in de cel (endocytose). Endocytose van de groeihormoonreceptor leidt tot vervoer naar en afbraak in het lysosoom, het afvalverwerkingsbedrijf van de cel. Het ubiquitinesysteem is betrokken bij dit proces. Ubiquitinering is het plakken van één of meerdere ubiquitinemoleculen aan een eiwit waarna het eiwit wordt afgebroken door het proteasoom, of wordt gesorteerd naar een bepaalde plek in de cel. Het ubiquitinesysteem herkent en bindt de groeihormoonreceptor aan het UbE-motif in het intracellulaire gedeelte van de groeihormoonreceptor. Na herkenning wordt de receptor naar het lysosoom gedirigeerd en afgebroken. Hoe dat precies verloopt is nog onduidelijk. We weten dat ubiquitinering van de receptor zelf hierbij niet nodig is. Wel is duidelijk dat er meerdere stappen nodig zijn voordat de receptor naar binnen kan.

In hoofdstuk II beschrijven we hoe het ubiquitineringseiwit βTrCP betrokken is bij groeihormoonreceptor endocytose. βTrCP is een onderdeel van een E3 ligase. E3 ligases binden specifieke substraten en plakken ubiquitinemoleculen aan deze substraten vast. Verlies van βTrCP resulteert in remming van groeihormoonreceptor endocytose en accumulatie van de receptor op het celoppervlak. Verder tonen we aan dat Cullin en Skp-1 (eveneens onderdeel van de ligase) samen met βTrCP in een complex aan de groeihormoonreceptor binden, waaruit blijkt dat βTrCP acteert als deel van een SCF-complex, een bekende E3 ligase. Bindingsproeven tonen aan dat niet het DSGXXS-motief, het algemeen bekende bindingsmotief van de SCF(TrCP) ligase, dat eveneens aanwezig is in het intracellulaire deel van de groeihormoonreceptor, bindt aan het WD40-domein van βTrCP. Tot onze verrassing bleek βTrCP te binden aan het UbE-motief van de groeihormoonreceptor, waarvan eerder al aangetoond was dat het essentieel is voor groeihormoonreceptor endocytose.

Hoofdstuk III beschrijft een andere belangrijke speler in groeihormoonreceptor endocytose, namelijk Jak2. We vonden dat Jak2, naast zijn functie als kinase, de groeihormoonreceptor endocytose remt. Deze functie is onafhankelijk van de kinase-activiteit van Jak2; binding van Jak2 aan de groeihormoonreceptor bleek voldoende om groeihormoonreceptor endocytose te remmen. Een verhoogde concentratie Jak2 zorgt dus voor accumulaetie van groeihormoonreceptoren op het celoppervlak. Aangezien βTrCP betrokken is bij de K48-ubiquitinering van de groeihormoonreceptor en de geaccumuleerde receptoren niet zijn geubiquitineerd, concludeerden we dat Jak2 vóór βTrCP acteert. Jak2 houdt de groeihormoonreceptor op het celoppervlak, totdat groeihormoon de receptor bindt en Jak2 zichzelf fosforyleert op tyrosine residu 119. Dit resulteert in loskoppeling van Jak2 van de groeihormoonreceptor. De receptor is dan in staat om βTrCP te binden en te endocyteren. In bepaalde celtypes detecteren we groeihormoon-geinduceerde groeihormoonreceptor endocytose. In muizenfibroblasten, die geen Jak2 kunnen maken, maten we constitutieve (groeihormoononafhankelijke) endocytose, totdat we Jak2 introduceerden Jak2. In dit modelsysteem hebben we laten zien dat zowel groeihormoon-afhankelijke als constitutieve groeihormoonreceptor endocytose op dezelfde manier endocyteren. Beide routes zijn afhankelijk van clathrine, het ubiquitinesysteem, βTrCP en het UbE-motief van de groeihormoonreceptor. Verder spelen het DSGXXS-motief en de tyrosineresiduën in het intracellulaire deel van de groeihormoonreceptor hierbij geen rol.

In hoofdstuk IV hebben we het eiwit βTrCP bestudeerd. βTrCP heeft twee isovormen: 1 en 2, die van verschillende genen worden afgeschreven; per isovorm komen meerdere splicevarianten voor. We bepaalden de localisatie van de isovormen en splicevarianten in de cel, de affiniteit voor twee verschillende substraten, de halfwaardetijd en de expressie in verschillende weefsels. Vooral de localisatie van de splicevarianten van βTrCP2 leidde tot interessante resultaten. Waar we splicevariant 2β alleen in de kern detecteerden, was splicevariant 2γ alleen detecteerbaar in het cytosol. Verder lieten we zien dat het substraatbindingsdomein (WD40) een belangrijke rol speelt bij de localisatie van βTrCP2. We vonden dat de groeihormoonreceptor, in tegenstelling tot β-catenine en de grote meerderheid van de βTrCP substraten, geen kinase-activiteit nodig heeft om βTrCP te binden: alleen de vrije βTrCP concentratie in de cel bepaalt de binding aan de groeihormoonreceptor. Dit is een gevolg van het feit dat het UbE-motief van de

groeihormoonreceptor geen serines bevat, terwijl het klassieke βTrCP-bindingsmotief, het DSGXXS-motief, twee serines bevat, die beide gefosforyleerd moeten zijn voordat βTrCP eraan kan binden. Verder hebben we gekeken naar de RNA-expressie van βTrCP splicevarianten in verschillende weefsels. De resultaten lieten zien dat de hoeveelheid mRNAs van de verschillende splicevarianten van zowel βTrCP1 en 2 in verschillende weefsels varieerde. Onze conclusie was dat deze regulatie van expressie van deze varianten leidt tot verschillen tussen cellen in verschillende weefsels.

In het laatste experimentele hoofdstuk V hebben we bepaald dat fosforylering van tyrosines 487, 534 en 627 belangrijk is voor groeihormoonreceptor signalering. De fosforylering van een of meer van deze aminozuren is voldoende om de STAT5B signalering aan te zetten na stimulatie met groeihormoon. Verder vonden we dat mutatie van tyrosine 627 naar een fenylalanine resulteerde in hyperfosforylering van de groeihormoonreceptor, Jak2 en MAP kinase, maar niet van STAT5B. Hoewel meer experimenten nodig zijn om het mechanisme van dit fenomeen te ontrafelen, is het waarschijnlijk dat tyrosine 627 een bindingsplaats is voor een proteine tyrosine fosfatase.

De bevindingen beschreven in de experimentele hoofdstukken II tot en met V in combinatie met de bestaande kennis samengevat in hoofdstuk I zijn samengevat in een nieuw model van groeihormoonreceptor endocytose (hoofdstuk 6, Fig. 1). De groeihormoonreceptor arriveert op het celoppervlak als een groeihormoonreceptor dimeer. Als de groeihormoonreceptor βTrCP bindt, wordt de receptor geselecteerd voor endocytose en wordt opgenomen in de cel (onafhankelijk van groeihormoon). Als daarentegen Jak2 bindt aan de groeihormoonreceptor, blijft de receptor op het celoppervlak totdat groeihormoonbinding resulteert in Jak2 fosforylering en signalering. Jak2 fosforylering leidt ook tot Jak2 dissociatie van de groeihormoonreceptor, waardoor βTrCP de groeihormoonreceptor kan binden en selecteren voor endocytose. Het is belangrijk vast te stellen dat beide routes hetzelfde endocytose mechanisme hebben. Het enige verschil is dat, als Jak2 gebonden is aan de groeihormoonreceptor, groeihormoon nodig is om Jak2 van de receptor af te krijgen. Uit dit model volgt dat de vrije concentraties van de belangrijke spelers voor groeihormoonreceptor endocytose, Jak2 en βTrCP, heel belangrijk zijn voor de regulatie van de hoeveelheid groeihormoonreceptoren aan het celoppervlak. Bij een relatief hoge Jak2 concentratie zullen meer (Jak2-gebonden) groeihormoonreceptoren aan het celoppervlak blijven dan bij een relatief lage concentratie Jak2. In feite bepaalt de vrije Jak2 concentratie de GH-gevoeligheid van cellen. Hieruit volgt ook dat cellen met veel Jak2 en weinig mRNAs voor groeihormoonreceptor lange tijd groeihormoon-ongevoelig zijn na een groeihormoonpiek vanuit de hypofyse, terwijl cellen met veel groeihormoonreceptor mRNA en weinig Jak2 hun gevoeligheid zullen behouden.

In dit proefschrift stond het onderzoek naar de regulatie van groeihormoonreceptorafbraak en signalering centraal. We hebben veel kennis vergaard over hoe de hoeveelheid groeihormoonreceptoren op het oppervlak van de cel geregeld wordt. Deze kennis is belangrijk voor het ontwikkelen van behandelingen voor aandoeningen waarbij de hoeveelheid groeihormoonreceptor op het celoppervlak ontregeld is. Geneesmiddelen die de binding tussen Jak2 of βTrCP en de groeihormoonreceptor kunnen voorkomen in patiënten met teveel of te weinig groeihormoonsignalering kunnen als behandeling dienen van patiënten met groeihormoonreceptor-geassocieerde aandoeningen.

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Joyce

Curriculum Vitae

Joyce Putters werd op 8 september 1981 geboren te Schelluinen. In juni 1999 behaalde ze haar VWO-diploma aan het gymnasium Camphusianem te Gorinchem. In september van datzelfde jaar begon ze aan de opleiding 'Biologie en medisch laboratoriumonderzoek' met specialisatie moleculaire biologie aan de Hogeschool van Utrecht. Haar onderzoeksstage vond plaats op de afdeling assay development en high-throughput screening van Johnson & Johnson, te Beerse, België. Onder begeleiding van Dr. Miroslav Cik en Martine Ercken werd een assay ontwikkeld om met high-throughput screening 'small molecules' te identificeren die de Tyrosine kinase B receptor kunnen activeren. Na het behalen van het diploma aan de Hogeschool van Utrecht in juni 2003 begon ze aan de masteropleiding 'Biomolecular Sciences' aan de Universiteit Utrecht. Haar onderzoeksstage deed ze in de vakgroep van Prof. Dr. Ger Strous op de afdeling celbiologie in het Universitair Medisch Centrum te Utrecht. Onderwerp was de groeihormoonreceptorgemedieerde celdood in humane kankercellen. In juni 2005 werd het masterdiploma behaald en in juli 2005 is ze begonnen als promovendus bij de vakgroep celbiologie in het UMC Utrecht onder begeleiding van Prof. Dr. Ger Strous. Het promotieonderzoek dat werd gedaan gedurende deze periode staat beschreven in dit proefschrift. Tijdens haar promotie heeft ze voor een periode van twee maanden onderzoek gedaan bij de vakgroep van Prof. Dr. Louis Muglia op de afdeling Pediatrics aan Washington University in St. Louis in de Verenigde Staten.