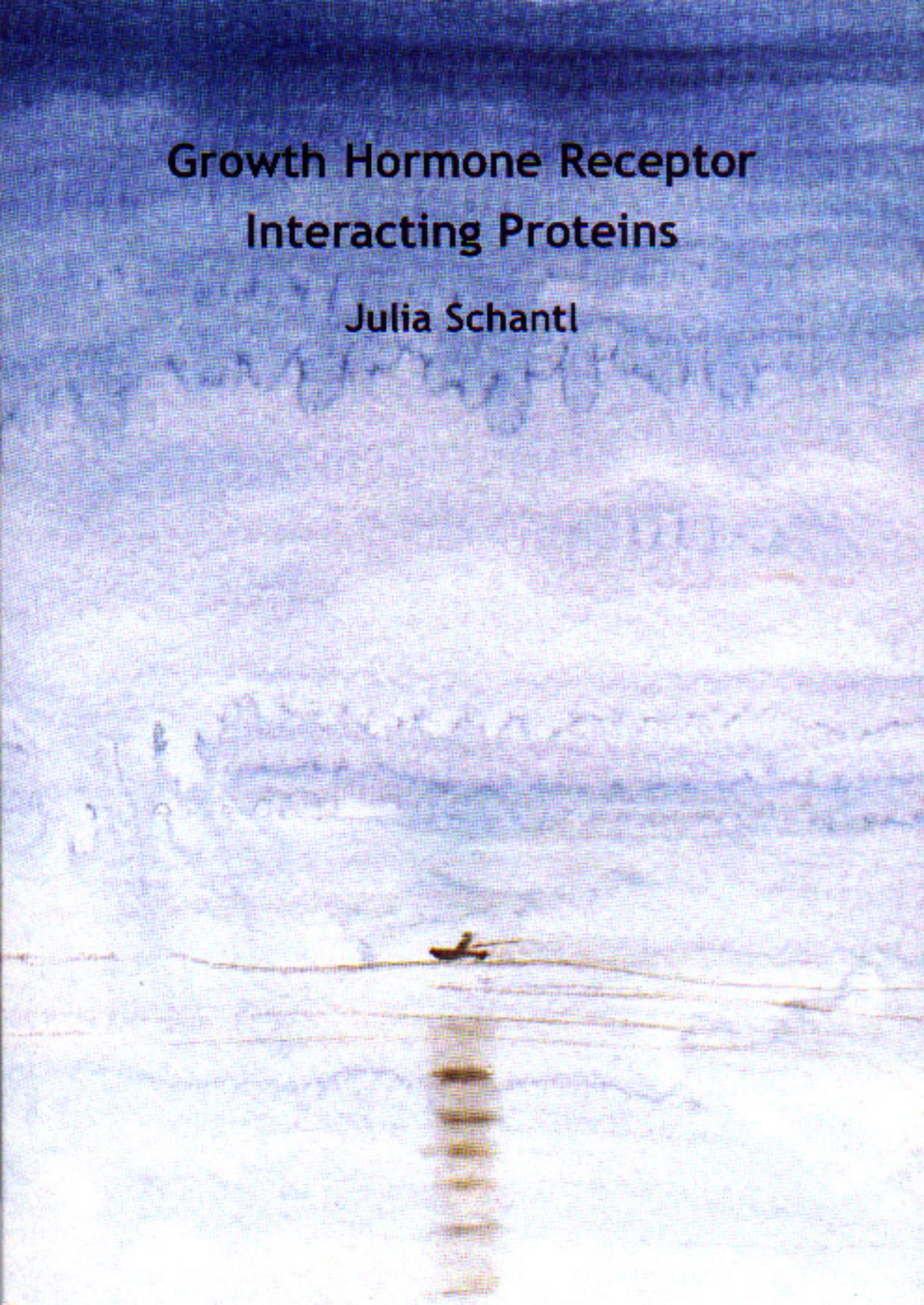
# Growth Hormone Receptor Interacting Proteins

Julia Schantl



# Growth Hormone Receptor Interacting Proteins

Cover: *Het Vissertje. Onderzoek naar de geheimen van het leven.*
Aquarelle by Leo Huijg, 2002.

Reproduction: Ponsen & Looijen, Wageningen

# Growth Hormone Receptor Interacting Proteins

Cellulaire eiwitten die interactie aangaan met de groeihormoonreceptor
(met een samenvatting in het Nederlands)

**Proefschrift**

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op dinsdag 21 januari 2003 des ochtends te 10:30 uur

door

Julia Angelika Schantl

geboren op 23 juni 1972 te Innsbruck, Oostenrijk

Promotor: Prof. Dr. G.J.A.M. Strous

Department of Cell Biology, University Medical Center and
Institute of Biomembranes, Utrecht University, The Netherlands

ISBN 90-393-3256-8

Printing of this thesis was financially supported by the 'J. E. Jurriaanse Stichting' and 'Dr. Ir. van der Laar Stichting'.

The research described in this thesis was performed at the department of Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands and was financially supported by a European Union Network grant (ERBFMRXCT96-0026) and by a grant of the Netherlands Organization for Scientific Research (NWO-902-23-192).

*Dedicated to the memory of Fabian Schantl*

# Contents

# Abbreviations

| | |
|---|---|
| ADAM | a disintegrin and metalloprotease |
| BAG | Bcl-2 associated anthogene-1 |
| Bcl-2 | B-cell lymphoma-2 |
| CHIP | C-terminus of Hsc70/Hsp70 interacting protein |
| DIABLO | direct IAP binding protein with low pI |
| DUB | deubiquitylating enzyme |
| DIAP | *Drosophila* inhibitor of apoptosis |
| ECD | extracellular domain |
| ER | endoplasmic reticulum |
| ESCRT | endosomal sorting complex required for transport |
| GH | growth hormone |
| GHBP | growth hormone binding protein |
| GHR | growth hormone receptor |
| HECT | homologous to E6-AP C-terminus |
| IAP | inhibitor of apoptosis |
| IGF | insulin–like growth factor |
| IGFBP | IGF binding protein |
| JAK | Janus kinase |
| MVB | multivesicular bodies |
| NF-κB | nuclear factor of κB |
| PMA | phorbol 12,13-myristate acetate |
| RGH | Reaper/Grim/Hid |
| RING | really interesting new gene |
| SMAC | second mitochondria-derived activator of caspases |
| SOCS | suppressors of cytokine signalling |
| STAT | transducers and activators of transcription |
| TACE | tumor necrosis factor-α converting enzyme |
| TMD | transmembrane domain |
| TNF | tumor necrosis factor-α |
| TGN | *trans*-Golgi netwerk |
| TPR | tetratricopeptide repeat |
| UbE | ubiquitin-dependent endocytosis |
| UIM | ubiquitin-interacting motif |

# Chapter I

## Introduction

Julia A. Schantl

Department of Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands

*Part of this chapter will appear as a review in Eur. J. Cell Biology*

# Contents Chapter 1

# Growth

Growth is one of the central research themes within the field of endocrinology. Many hormones and growth factors are involved, directly or indirectly, in the regulation of expansion of bone, organ and other tissues. Experiments by Evans and Long in the beginning of the 20th century have demonstrated the existence of growth promoting proteins in the anterior pituitary [1]. Later studies established that growth retardation in hypophysectomized rats could be reversed by pituitary implants [2]. This led to the investigation for growth promoting factors in the anterior pituitary, followed by the identification of bovine growth hormone (bGH) in 1945 [3]. Because bGH failed to promote human growth [4], the isolation of human growth hormone (hGH) was undertaken and succeeded in 1956 [5]. In clinical trials, both hGH and monkey GH stimulate longitudinal growth [6]. Biochemical differences between primate and non-primate GH account for the species specificity of the physiological effects [6]. Subsequently, evidence accumulated that pituitary-derived GH does not act directly on its target tissues to promote growth but rather through factors termed somatomedins or insulin-like growth factors (IGFs) [7]. In 1980, D'Ercole and co-workers discovered that IGFs are expressed not only in hepatic tissue but also in adipose, lung, kidney, heart, bone, and skeletal muscle, leading to the hypothesis that IGF acts in an autocrine/paracrine manner in diverse tissues [8]. Furthermore, circulating IGF is not required for stimulation of longitudinal bone growth, but rather GH itself directly stimulates the differentiation of chondrocyte precursor cells while IGF enhances the clonal expansion of differentiated chondrocytes [9]. This led to a new concept known as the dual effector hypothesis [10]. At present, it is assumed that GH can act in an endocrine way via circulating IGFs as well as in an autocrine/paracrine way involving locally expressed IGFs [11].

The effectiveness of the endocrine system relies on the specific interaction of a hormone with its receptor. GH exerts its action via growth hormone receptors (GHR) on the cell surface of its target cells. The GHR has been the first member of the cytokine receptor family to be characterized. The receptor is present throughout the body with highest expression levels found in liver and adipose tissue. Upon binding of GH, a complex signaling cascade is activated that triggers signal transduction in the nucleus. The GH-GHR complex is internalized and degraded.

Beside growth, GH regulates lipid, carbohydrate, nitrogen and mineral metabolism within a cell through its interaction with GHR [12,13]. The interplay between GH and

GHR needs to be tightly balanced. Disturbance of this equilibrium or defects in either GH or GHR result in growth disorders and contribute to severe pathological defects such as diabetes mellitus, cardiovascular malfunction, and immunodeficiency syndromes [14,15]. Understanding of the underlying molecular mechanisms of GH action can help to develop new therapeutics to treat these disorders.

## Growth Hormone

The growth hormone, also referred to as somatotropin or somatotrope hormone, is the most abundant of all anterior pituitary hormones and belongs to a family of hormones that have evolved from a common precursor [16]. Additional members of this family are prolactin (PRL), placental lactogen (PL) and the recently identified hormones proliferin, proliferin-related protein, prolactin-like protein, and somatolactin [17]. GH contains two disulfide bonds and four α-helices. These secondary structures adopt an up-up-down-down conformation [18] similar to the conformation of other cytokines, even though there is little sequence similarity between cytokines. Human GH is a protein of 191 amino acids that is produced and released by the anterior pituitary gland to circulate as an endocrine hormone. GH synthesis is reported in a number of extra-pituitary tissues, suggesting that GH has a local paracrine/autocrine effect [19]. Secretion of GH from the pituitary is directly regulated by the hypothalamus, which produces the inhibitory factor somatostatin (also known as somatotrope release-inhibitory hormone or SRIH) and the stimulating factor GH-releasing hormone (GHRH) [20]. The interplay between these two effectors of the GH secretion leads to a pulsatile GH secretion pattern [21]. This is important for both growth and metabolic actions, which are dependent on the pattern of GH exposure, implying that signaling is both concentration- and time-dependent [22]. GH mediates IGF-I expression and secretion in different tissues like liver, adipose, kidney, lung, heart, and skeletal muscle with most of the circulating IGF-I being from hepatic origin [23]. IGF-I is present in blood and tissue, free and bound to IGF-binding proteins (IGFBPs) [24] and acts on target tissue via the IGF receptor [25]. Circulating IGF-I operates in a negative feedback loop to inhibit GH secretion in peripheral tissues [26] as well as at the level of the pituitary [27] and possibly at the level of the hypothalamus [28].

Recently, an alternative physiological system for the regulation of GH secretion has been identified by the finding that synthetic GH releasing peptides (GHRP) are capable to release GH from the pituitary in a dose-dependent manner [29,30]. Soon it became

apparent that different mechanisms are utilized by GHRHs and GHRPs resulting in GH secretion. This has led to the identification of the GH secretagogue receptor (GHS receptor) [31]. Finally, the endogenous ligand of GHS receptor, ghrelin, has been identified from stomach extracts [32].

The concentration of GH is also influenced by factors like gender, body composition, and exercise. Secretion declines during normal aging. Many age-related changes, like osteoporosis and muscle atrophy, may be partly due to the decreased actions of GH and IGF-I [33].

## Growth Hormone Physiology

Postnatal longitudinal body growth and differentiation of muscle, bone, and cartilage cells involves the regulation by GH [34]. Through the interaction with GHR, GH also regulates the lipid, carbohydrate, nitrogen, mineral metabolism, and electrolyte balance within a cell [12,13]. For example, recombinant human GH administrated to older men (and recombinant porcine GH administrated to pigs) increases lean body mass and decreases fat [35]. GH induces two opposite actions on carbohydrate metabolism [12]. First, the initial insulin-like effect involves lipogenesis and enhances glucose and amino acid metabolism. Second, after several hours, the anti-insulin effect occurs and results in lipolysis, hyperglycemia, and hyperinsulinemia. Chronic elevations of GH levels, as seen in acromegalic patients, often lead to insulin resistance and in many cases to the onset of diabetes [36,37]. The anabolic effects of GH result in a rise of protein synthesis reflected by an increase in lean body mass and a reduction of body fat [38,39]. Based on the anabolic nature of GH, it is used to counteract the decrease in body mass observed in patients due to surgery [40], severe burns [41], or ‘wasting’ diseases like AIDS or cancer [42]. GH also plays an integral role in the development and maintenance of the immune system [43,44]. Through direct actions on the brain, GH may also modulate emotions, stress response, and behavior [45].

Hyposecretion of GH during postnatal development gives rise to dwarf phenotype, whereas hypersecretion of GH before puberty leads to gigantism and acromegaly in adults [46]. Acromegaly causes enlarged bones, especially in the face, hands, feet, and fingers, as well as oversized organs such as the thyroid gland. In addition, hypersecretion can result in impaired cardiovascular function, and metabolic disorders like glucose intolerance and diabetes mellitus [15].

# Growth Hormone Receptor

## Cytokine/Hematopoietin Receptor Superfamily

The GHR is a member of an extended family of hormone and cytokine receptors, referred to as the cytokine/hematopoietin receptor superfamily [47]. Members of this family include receptors for GH, prolactin (PRL), erythropoietin (EPO), interleukin (IL) 2-7, 9, 11-13 and 15, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), cilliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and mpl ligand [48,49]. These receptors are called class I cytokine receptors, whereas receptors for interferon (IFN) $\alpha/\beta$, IFN$\gamma$, and IL-10 are more distantly related and are considered class II cytokine receptors [50].

The class I superfamily of cytokine receptors shows limited amino acid homology (14–44%) in the region spanning approximately 210 amino acids in the extracellular domain (corresponding to two fibronectine III domains in similar size) with two conserved pairs of cysteine residues in the extracellular domain and a conserved tryptophan residue adjacent to the second cysteine in the N-terminal fibronectin domain, and a WSXWS-like motif (single letter amino acid code; X represents any amino acid) in the C-terminal fibronectin domain. From the seven cysteines that are present in the extracellular domain of the GHR six cysteines are linked by disulfide bonds (Figure 1). The WSXWS-like motif in the GHR is structurally homologous but differs in amino acid composition: YGEFS (single letter amino acid code; X represents any amino acid). This motif plays an important role in ligand binding, but no direct interaction with GH has been observed [18]. Mutations of the tyrosine or serine residues result in impaired binding affinity and trafficking of the receptor, probably through structural disturbance in the extracellular domain [51].

Cytokine receptors possess no canonical tyrosine kinase consensus sequence in their cytosolic tail. Instead, they interact with cytosolic tyrosine kinases of the Janus kinase (JAK) family, which consists of JAK1, JAK2, JAK3 and Tyk2 [48,52]. The GHR interacts predominantly with JAK2 [53], but studies in cell lines show that GH also induces phosphorylation of JAK1 [54] and JAK3 [55]. Tyk2 associates with GHR in human liver cells [56], but GH does not cause detectable tyrosine phosphorylation and activation of Tyk2 in IM-9 cells [57]. Recent studies have demonstrated that a specific cytokine receptor can activate several JAKs and that this activation may differ between

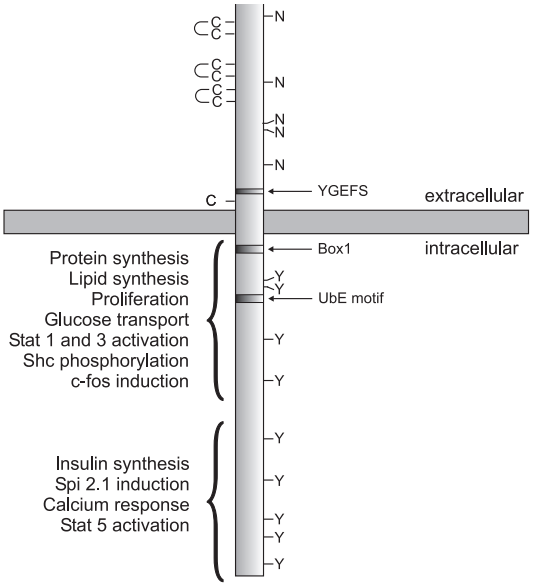



**Figure 1. Schematic representation of the GHR.**
The GHR is a type I membrane protein and consists of an extracellular domain, a single transmembrane domain, and a cytosolic region. The extracellular domain contains five potential N-linked glycosylation sites (N), seven cysteine residues (C), and the YGEFS motif at the indicated positions. In the cytosolic region, a proline-rich region required for signal transduction (Box1) and the ubiquitin-dependent endocytosis motif (UbE motif) are shown. Furthermore, rabbit GHR contains nine intracellular tyrosine residues (Y), which can be phosphorylated upon GHR activation.

tissues. Experiments by Hellegren *et al.* [58] show that GHR predominantly interacts with JAK2 in rat liver tissue, whereas in adipose tissue at least one third of the associated JAK species is JAK1, indicating that tissues differ in JAK specificity.

A conserved cytosolic proline-rich region, referred to as Box1, is required for binding of JAK2 to the GHR [59,60]. Box1 is situated in close proximity to the membrane and is eight amino acids in length with a consensus sequence of ψXXXAPXP (ψ, hydrophobic amino acid; X, any amino acid; A, aliphatic amino acid; P, proline) [48,49]. In mammalian GHR, Box1 is ILPPVPVP (I, isoleucine; L, leucine; P, proline; V, valine) [49]. The second conserved but less defined motif in cytokine receptors is referred to as Box2. This motif begins with a cluster of hydrophobic amino acids interspersed with acidic amino acids and ends with one or two positively charged amino acids [61]. In the GHR, Box2 begins approximately 30 amino acids C-terminal to Box1 and spans about 15 amino acids [62]. Mutational analysis suggests that Box1 and Box2 play critical roles in signal transduction mediated by members of the cytokine receptor superfamily [63,64].

## Growth Hormone Receptor Structure

Cloning of the GHR from rabbit and human liver cDNA libraries in 1987 opened the door to the study of molecular mechanisms of GH [65]. Subsequent cloning of the

GHR of rat, cow, mouse, sheep, pig, and chicken has revealed an amino acid sequence homology of more than 50% between the different species [50]. The cDNA of human as well as rabbit GHR encodes for a 638 amino acid protein, which includes an 18 amino acid signal peptide and a 620 amino acid mature GHR protein. The GHR is a type I transmembrane protein, with an extracellular domain of 246 residues, a single membrane spanning domain of 24 residues, and 350 intracellular residues. Based on the amino acid sequence, the predicted molecular weight is 70 kDa; however, the observed molecular weight based on gel electrophoresis is 109 kDa in human IM-9 cells [66] and 124 kDa in human liver cells [67]. The discrepancy may be explained by posttranslational modification, such as N-glycosylation [68] and covalently bound ubiquitin [65], a 76 amino acid protein. Glycosylation appears neither necessary for ligand binding [69] nor for correct biosynthetic processing [70].

The GHR gene spans at least 87 kbp and is composed of at least 10 exons [71]. The presence of alternatively spliced mRNAs, potentially encoding short isoforms of the GHR, has been reported in various tissues and cultured cells [72,73]. One well-characterized isoform of the GHR is GHRd3, lacking exon 3 and therefore missing 22 N-terminal amino acids [74]. However, ligand binding and internalization are not affected [75,76]. Alternatively spliced variants that result in truncations of the cytoplasmic domain have been identified in patients with short statue [77]. These GHR truncations have a severe effect on signaling and internalization [73]. In addition to splice defects, mutations that affect GH binding or anchoring of GHR in the membrane have been reported [78]. These mutations result in a dwarf phenotype, referred to as Laron's syndrome [79].

## Growth Hormone Binding Protein

The growth hormone binding protein (GHBP) is one of the best-studied examples of a large family of soluble, circulating ectodomains of cytokine receptors [80]. It has been characterized in numerous species and has been attributed to diverse physiological roles, none of which are proven. GHBP is complexed to approximately 50% of the circulating GH, thus creating a GH reservoir [81]. Thereby, GHBP protects GH from degradation and excretion, prolongs its half-life, and may enhance its bioactivity through these mechanisms [82]. GHBP may also act as an inhibitor of GH action at the tissue level by competition with GHR for its ligand GH and by forming unproductive GHR-GHBP heterodimers that are unable to signal [83]. Furthermore, GHBP plasma levels are thought to reflect GHR abundance in the whole body, particularly in liver. Finally, the

generation of GHBP by proteolysis represents one of the mechanisms for down-regulating the GHR availability [84].

Different mechanisms are employed in various species to generate GHBP. In several rodents (e.g. rats and mice), GHBP is encoded by an mRNA originating from alternatively spliced mRNA of the GHR and contains a short hydrophilic C-terminal extension that is not found in the membrane receptor [85,86]. This hydrophilic tail is encoded by a special exon (8A) interposed between exons 7 (extracellular) and 8 (transmembrane domain) [87]. Many other species miss exon 8A, and the GHBP is generated by proteolytic cleavage from the membrane bound GHR, a process also known as shedding [88]. The cellular location (plasma membrane or intracellular compartments) where the cleavage occurs is not known with certainty. The fact that a truncated form of GHR (GHR 1-279) with a prolonged residence time at the plasma membrane is particularly prone to GHBP shedding suggests that the cleavage takes place at the cell surface and that the cytosolic tail of the GHR is not necessary for the proteolytic cleavage of GHBP [72]. Shedding of GHBP can be inhibited to some degree with EDTA and certain protease inhibitors. These findings imply a divalent metal dependence of the proteolysis [89]. Production of GHBP can be stimulated by phorbol 12,13-myristate acetate (PMA), growth factors [90], and alkylating agents like N-ethyl-maleimide [91]. These observations implicate protein kinase C in the pathway resulting in GHBP shedding [92] and led to the speculation that the unpaired cysteine (C 241) of the GHR is involved in the cleavage process. However, mutational analysis does not support this model [93]. Furthermore, GH inhibits PMA induced GHR proteolysis by inducing a conformational change that renders the receptor protected from proteolysis [94]. The protease responsible for GHR cleavage in response to phorbol ester treatment has recently been identified as TACE or ADAM-17 [95] (see section ‘GHR INTERACTING PROTEINS’, p. 44). The exact mechanism by which the sheddase recognizes the GHR is still unknown. Also, sequence comparison of GHRs that are prone to shedding and those that are not cleaved yielded no clue as to the cleavage site. Recently, a three amino acid juxtamembrane region in the extracellular domain has been reported to be a structural determinant required for shedding [96]. Preliminary experiments have identified a murine GHR cleavage site nine residues N-terminal to the first intramembranous amino acid [97].

# Growth Hormone Signalling

## Growth Hormone Binding and Receptor Dimerization

The first step in GH signaling is the binding of GH to its receptor. Crystallographic and size-exclusion chromatography studies of the extracellular domain of the GHR have revealed a trimeric complex of two receptor molecules with one single hormone molecule [18,83]. Furthermore, mutagenesis strategies have been employed to define the binding determinants in the GH and the extracellular domain of the GHR [98,99]. Two distinct binding sites have been identified in GH with slightly different binding affinities [100]. Due to the different affinities, the $GHR_2$-$GH_1$ complex formation has been proposed to occur sequentially: first, GH binds to the receptor with the high affinity binding site 1, and second, the contacts at binding site 2 of GH with the second GHR molecule and a contact region between the two extracellular domains of the receptor molecules stabilize the binding of the second receptor [83]. Mutation of binding site 2 of human GH (G120R) disrupts the binding at site 2 [101]. The mutant GH fails to induce GHR phosphorylation and GH stimulated cell proliferation suggesting that binding of a GHR at binding site 2 of GH is required for GH signaling [69].

Based on these crystallographic data, it was postulated that a single GH molecule dimerizes two GHR molecules through interactions between the membrane-proximal extracellular regions of adjacent GHR molecules, referred to as subdomain 2 [18]. Mutations of conserved amino acids in this domain disrupt ligand-induced signal transduction presumably by preventing receptor dimerization [102]. However, dimerization itself is not sufficient for signal transduction because administration of monoclonal antibodies directed against the extracellular domain of the GHR resulted in dimerized GHRs but failed to induce signal transduction. The GHR extracellular domain contains two subdomains, which are separated by a hinge region [18]. Mellado *et al.*, who developed a monoclonal antibody against the extracellular hinge region of the GHR [109], showed that antibody binding to the cell surface receptor increased upon GH binding, but not when the GH (G120R) was used [103]. This suggests that signal transduction requires a specific orientation of two GHR molecules. In recent years, evidence accumulated, that GHR already exists as a preformed dimer at the cell surface. Cross-linking studies with $^{125}$I-labeled GH or GH antagonist B2036, which contains one defective GHR binding site, revealed complexes similar in size that correspond to a $GHR_2$-$GH_1$ complex [104,105]. Further evidence was provided by the finding that

immunoprecipitation of a full-length GHR resulted in co-immunoprecipitation of a truncated receptor that was not recognized by the antibody used in the immunoprecipitation [106]. This interaction most likely reflects dimerization. Larger complexes, containing more than two GHR molecules, have not been observed. Strikingly, dimerization was not only observed between mature, glycosylated forms of the receptor but also between precursor species, which reside in the ER [106]. These findings indicate that receptor dimerization already occurs in the ER and thus is independent of ligand binding. How the dimerization of the GHR takes place in the ER is still unknown, but there are indications that neither the transmembrane domain (TMD) nor the cytosolic domain of the receptor are involved in dimerization. Elimination of 97% of the cytoplasmic tail of the GHR does not effect the heterodimerization of the truncated GHR with full length GHR [72]. Furthermore, mutating single and multiple amino acids of the TMD to alanine or the replacement of the GHR TMD by a heterologous TMD does not disrupt GHR dimerization [409]. That leaves the extracellular domain (ECD) as an important factor in receptor dimerization. Amino acids of the membrane proximal subdomain 2 of the extracellular domain do not effect dimerization [409], whereas the replacement of the entire ECD with part of the LDL receptor related protein (LRP) results in monomeric chimers [409]. On the other hand, the ECD is not required to maintain the GHR in the dimerized state, because protease-digestion of the cell surface localized GHRs, which are already dimerized, does not disrupt dimerization of the membrane bound remnant protein [106]. Therefore, the ECD might be involved in the initial dimerization process in the ER, whereas the TM domain is sufficient to maintain the GHR dimerized.

Although GH is not required for dimerization, GH binding is essential for signal transduction. The activation of GHRs upon GH binding probably results in a confirmational change of the receptor, leading to signal transduction events inside the cell. Mutations of amino acids of subdomain 2 prevent the reorganization induced by GH and result in signaling deficient mutants [409]. These results link subdomain 2 to signal transduction rather than dimerization.

## Growth Hormone-Dependent Signal Transduction

GH binding to the GHR promotes tyrosyl phosphorylation in the receptor [107] and other cellular proteins [108-111]. Tyrosine kinase activity capable of phosphorylating the GHR was found in a highly purified preparation of the GHR suggesting that a tyrosine kinase is involved in mediating the actions of GH [112]. However, sequence analysis of

GHR cDNA revealed no homology to known tyrosine kinases [65]. These two contrasting observations were reconciled by the finding that in response to GH the non-receptor kinase JAK2 is rapidly activated and becomes tyrosyl phosphorylated [53]. The membrane-proximal proline-rich region of the GHR is essential for JAK2 association and activation [113]. In response to GH binding, the affinity of JAK2 to the GHR appears to increase. Mutation or deletion of the proline-rich region, known as Box1, abolishes JAK2 binding as well as GH-induced proliferation [34].

Upon conformational change as a result of ligand binding to the preformed GHR dimer, GHR-associated JAKs are spatially positioned and/or conformationally modified resulting in trans-phosphorylation of one or more tyrosines and catalytic activation of the kinase domains of the paired JAK2. In turn, the activated JAK2 phosphorylates the intracellular domain of the GHR. Both the phosphorylated receptor and JAK2 provide docking sites for a variety of signaling molecules that contain Src homology 2 (SH2) or other phosphotyrosine-binding (PTB) motifs, thereby activating specific signaling

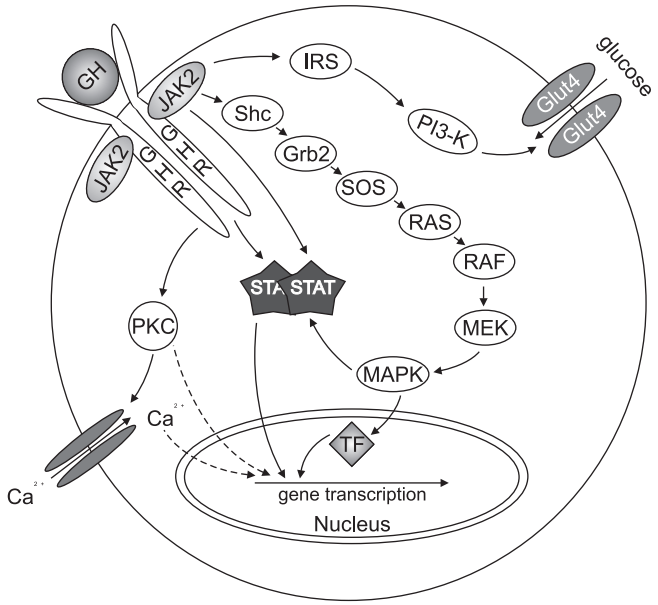



**Figure 2. Schematic representation of the signaling pathways activated by GH.**
In response to GH binding to the GHR, JAK2 is activated and tyrosyl phosphorylated. In turn, JAK2 activates the STAT, MAPK, IRS and/or PKC pathway, which ultimately results in the activation of gene transcription. See text for details.

pathways [Figure 2 and ref. 114]. Among the activated proteins are members of the signal transducer and activator of transcription (STAT) family that couple ligand binding to cellular receptors with the activation of gene transcription [115]. GH-activated JAK2 phosphorylates at least four members of the STAT family (STATs 1, 3, 5A, and 5B) on their conserved C-terminal tyrosine, leading to their dimerization, nuclear localization, DNA binding, and activation of transcription [116]. A second important pathway is the Ras-MAP (mitogen-activated protein) kinase pathway. GH activates this pathway by JAK2 phosphorylation of SHC, followed by the sequential recruitment of Grb2, Son-of-sevenless (Sos), Ras, Raf, and MAP kinase kinase (MEK), and ultimately results in the activation of the MAP kinases ERK1 and ERK2 [117]. MAP kinases are a family of serine/threonine/tyrosine kinases that mediate both cellular growth and differentiation upon stimulation by many growth factor receptors and hormones [118]. GH binding also results in tyrosine phosphorylation of insulin receptor substrate (IRS)-1, IRS-2, and IRS-3 [119-121]. These molecules have been identified as signaling molecules for insulin and the closely related IGFs, and are therefore proposed to mediate the insulin-like effects of GH. Phosphorylated IRS proteins associate with the 85 kDa regulatory subunit of phosphatidylinositol (PI)-3-kinase and the tyrosine phosphatase SHP-2 [122]. PI-3-kinase has been implicated in GH-induced insulin-like effects, and its activation leads to translocation of glucose transporter 4 (GLUT4) to the plasma membrane that ultimately stimulates glucose uptake [123]. Another pathway activated by GH involves the activation of protein kinase C (PKC) through phospholipase C (PLC) [124]. Activation of PKC by GH is thought to stimulate lipogenesis [125-127], induce c-fos expression, and increase intracellular $Ca^{2+}$ levels by activating L-type calcium channels [126,127]. Intracellular calcium is essential for GH-induced transcription of the *spi 2.1* gene and the effects of GH on the metabolism in adipocytes [128]. Regulation of calcium influx via L-type calcium channels is the only event, which is independent of an intact Box1 or JAK2, but requires the C-terminal cytosolic tail of the GHR [128].

## Negative Regulators of Growth Hormone Signaling

Receptor signal transduction requires the precise control of the magnitude and duration of a signal. After GHR activation, GH-induced signal transduction is terminated as a result of the initiation of negative regulator pathways. At least two families of proteins inhibit the JAK/STAT signaling pathway: phosphatases and suppressors of cytokine signaling (SOCS).

Tyrosine phosphatases contribute to the signal down-regulation by

dephosphorylation of JAK2. The SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1) is activated by GH and associates with phosphorylated JAK2 [129]. GH also induces nuclear translocation and binding of SHP-1 to tyrosyl phosphorylated STAT5b, suggesting that this GH-activated phosphatase is required for dephosphorylation and deactivation of nuclear STAT5b. SHP-2 binds to tyrosine residues in the C-terminus of the GHR [130]. Mutations of this tyrosine residues or deletion of the C-terminal region result in the prolonged activation of the GHR, JAK2 and STAT5b [131,132]. SHP-2 can also function via an ancillary signal-regulatory protein (SIRP) α1. After GH-induced tyrosyl phosphorylation, one or more SHP-2 molecules can be recruited by SIRPα1 [133]. Interaction between SIRPα1 and SHP-2 enhances the phosphatase activity of SHP-2 [134] and results in the dephosphorylation of JAK2, SIRPα1 and possibly GHR [135].

Recently, a new class of proteins termed suppressors of cytokine signaling (SOCS) have been implicated in negatively regulating cytokine signaling [136]. The SOCS family comprises eight proteins, SOCS 1-7 and cytokine inhibitor of signaling (CIS) that contain a variable N-terminus, a central SH2 domain, and an approximately 40 amino acid C-terminal region of homology termed SOCS-box [137]. SOCS expression is induced by GH activation of the JAK/STAT pathway and inhibits the JAK/STAT pathway either by direct interaction with activated JAK or with GHR [138,139]. Structural and functional studies demonstrate that the SH2 domain and the N-terminal region of SOCS-1 are essential for inhibiting JAK activity, whereas the C-terminal SOCS box is not required [140]. The function of the C-terminal SOCS-boxes remains unclear. An alternative model of SOCS function has been proposed, based on the observation that the conserved SOCS-box binds to the elongin B/C complex [141]. These elongins also interact with Cullin-2, a putative ubiquitin ligase, and thereby mediate the ubiquitination and subsequent degradation of the SOCS protein and its interacting molecules like JAK2 [142]. Consistent with this model, proteasome inhibitors prolong phosphorylation of JAK2 and the GHR [131].

# GH, GHR and Apoptosis

## Apoptosis

Apoptosis, also known as programmed cell death, is a crucial process during embryonic development and normal tissue [143-145]. Cell death occurs under a variety

of physiological and pathological conditions, and deregulation of this process plays a significant role in the pathogenesis of several diseases [146,147]. Diminished apoptosis has been linked to auto-immune syndromes and is thought to be important for both the development of tumors and resistance to chemotherapy [148]. On the other hand excessive apoptosis has been implicated in neurodegenerative diseases [149].

Apoptosis is designed to eliminate excessive or harmful cells in a highly regulated manner without harming neighboring cells. It is characterized by chromatin condensation at the periphery of the nucleus, organelle breakdown, and plasma membrane blebbing, followed by the disintegration of the cells into multiple membrane enclosed vesicles, some of which contain nuclear fragments [146]. These membrane-enclosed vesicles are rapidly ingested by neighboring cells [150]. The process of apoptosis can be triggered by various extracellular and intracellular signals, which results in the transduction of the signal by a conserved set of proteins. Ultimately, this leads to the activation of a family of proteases, the cysteine-dependent aspartate specific proteases (caspases) that share the ability to cleave their substrates at the caryl side of aspartate residues. Caspases are initially synthesized as pro-enzyme, termed procaspase, with little or no enzymatic activity. Procaspases are cleaved by an upstream protease, often also a caspase, or by an increase in local caspase concentration that leads to a cleavage-independent activation [151-153]. Caspases are thought to target key proteins in the cell, such as nuclear lamins. Furthermore, caspases are involved in the activation of endonucleases, which cleave DNA at internucleosomal sites into fragments, typically 180 bp, resulting in DNA laddering characteristic for apoptosis. Once initiated, the activation of this proteolytic cascade is irreversible, and consequently, strict regulation of this process is indispensable.

Two principle pathways can initiate apoptosis: (1) the death receptor or extrinsic pathway, and (2) the mitochondrial or intrinsic pathway (Figure 3). The extrinsic pathway is activated by a receptor-initiated pro-apoptotic signal. Receptors like TNF receptor 1 or Fas interact with their cognate ligands, which results in the recruitment of the death-inducing signaling complex (DISC) to the Fas cytoplasmic domain [154]. The DISC complex contains adaptor proteins, such as Fas-associated death domain (FADD)/Mediator of receptor-induced toxicity (MORT1), that assist in the recruitment of procaspase-8. Recruitment of procaspase-8 results in cross-activation of caspase-8 and release of active caspase-8 into the cytosol [155-157]. Subsequently, caspase-8 cleaves and activates procaspase-3, resulting in initiation of a caspase cascade through caspase-3. Therefore, caspase-8 is often referred to as an initiator caspase, whereas caspase-3 is

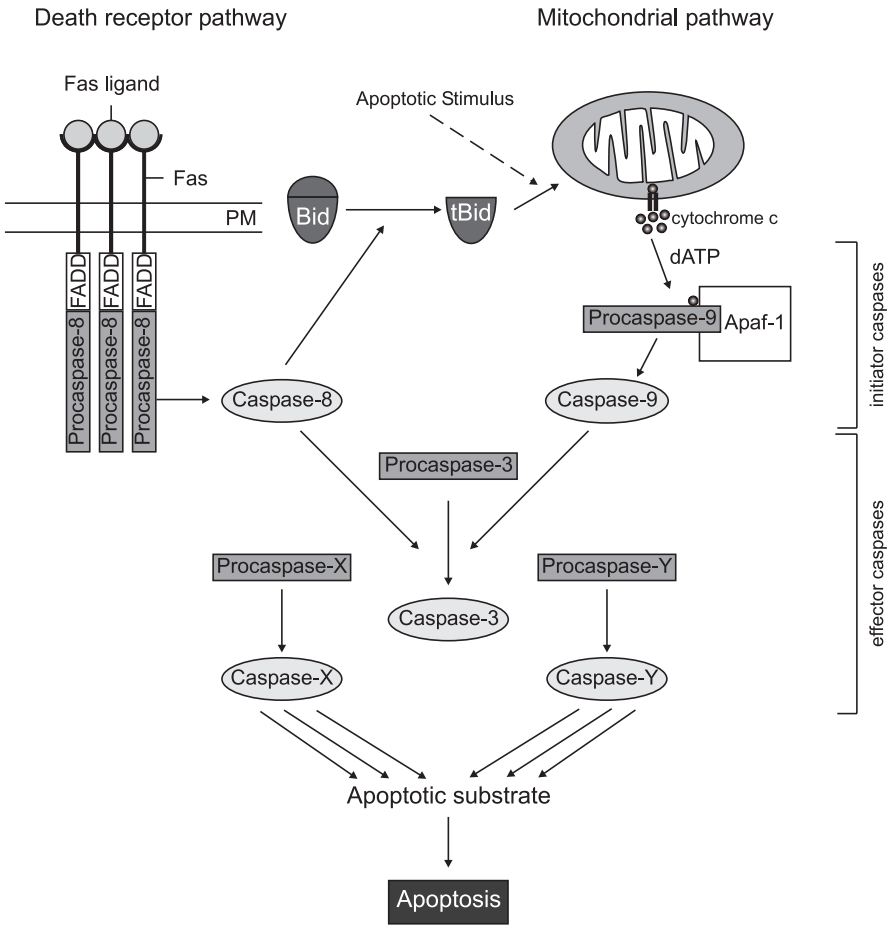



**Figure 3. Schematic representation of apoptotic signaling pathways.**
Two different pathways can induce apoptosis: (1) the death receptor pathway, and (2) the mitochondrial pathway, each of which involves the activation of specific initiator caspases, such as caspase-8 and caspase-9. Both routes eventually converge at the level of caspase-3 activation. Downstream of caspase-3 several signals serve to mediate apoptosis. See text for details.

considered to be an effector or executioner caspase [152].

The mitochondrial apoptosis pathway is stimulated by stress-induced signals, such as DNA damage, growth factor deprivation, or developmental apoptotic triggers. This process is thought to be triggered by translocation of Bax and Bak from the cytoplasm to mitochondria. Bax and Bak are pro-apoptotic members of the B-cell lymphoma-2 (Bcl-2) family comprising proteins that can both block and enhance apoptosis. Bax and Bak

oligomerize and insert into the outer mitochondrial membrane, where they participate in the formation of pores and facilitate the release of cytochrome c [158-162]. At the same time other pro-apoptotic factors, like apoptosis-inducing factor (AIF) and endonuclease G exit from the mitochondrial intermembrane space [163]. Endonuclease G has been implicated in apoptotic DNA degradation [164]. Cytochrome c binds to the adaptor molecule apoptotic protease-activating factor 1 (Apaf-1) in the presence of ATP or dATP. This complex, termed apoptosome, recruits and activates procaspase-9 [165-168]. In turn, caspase-9 can activate caspase-3, which is again responsible for initiating a caspase cascade, ultimately resulting in cell death.

The two pathways described above are not strictly separated. For example, receptor-initiated cell death can initiate the mitochondrial pathway by the use of intermediates such as Bid. After truncation of Bid (tBid), tBid translocates to the mitochondria and triggers the intrinsic pathway [159,161].

## Regulation of Apoptosis

The first level of caspase regulation involves the conversion of pro-caspases to their active forms in response to apoptotic stimuli. Bcl-2 family members are the key regulators that control of cytochrome c release and other apoptosis promoting factors from mitochondria. The second level of regulation involves the specific inhibition of active caspases by inhibition of apoptosis proteins (IAPs).

### *Bcl-2 Family*

The B-cell lymphoma-2 (Bcl-2) family encompasses both anti-apoptotic and pro-apoptotic members. At least 15 Bcl-2 family members have been identified in mammalian cells and several others in viruses [148]. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1–BH4). Most anti-apoptotic members like Bcl-2 or Bcl-$x_L$ share sequence conservation throughout all four BH domains. Pro-apoptotic members can be subdivided into two distinct groups: (1) more fully conserved, multi-domain members such as Bax and Bak, show homology in BH domains 1–3 [169], whereas (2) Bid, Bik, Bim, Bad, and Noxa contain only a conserved BH3 domain (BH3-only proteins), which serves as the critical death domain [170]. Most Bcl-2 family members possess a C-terminal hydrophobic tail, which facilitates their interaction with the endoplasmic reticulum, the nuclear envelope, and the mitochondrial membrane. The membrane of the mitochondria is the place where the anti-apoptotic members normally reside and where many pro-apoptotic members assemble

during apoptosis [171].

The Bcl-2 family members are involved in the mitochondrial apoptosis pathway, in which they regulate cytochrome c release [172]. The current model implicates that 'BH3-only' proteins activate the multi-BH-domain pro-apoptotic members Bax and Bak to trigger the mitochondrial pathway [158,173]. This results in cytochrome c release to activate caspase-9-dependent apoptosis. The anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-$x_L$ seem to function primarily as suppressors of the pro-apoptotic function of apoptogenic Bcl-2 family members. By sequestering BH3-only proteins in stable mitochondrial complexes, Bcl-2 and Bcl-$x_L$ are thought to prevent the allosteric activation of multi-BH domain apoptogenic Bcl-2 members and the subsequent mitochondrial program of apoptosis [174].

### *Inhibitor of Apoptosis (IAP)*

Inhibitor of apoptosis (IAP) proteins were first identified as viral products of baculovirus that inhibit the apoptotic defense mechanisms of the host cell and thereby providing the virus sufficient time to replicate [175,176]. IAP homologues have been identified in a wide range of eukaryotic species as diverse as yeast, nematodes, flies, and humans [177-180]. They are defined by the presence of one or more characteristic baculovirus IAP repeat (BIR) and by their ability to inhibit apoptosis [181,182]. The BIR motifs are essential for the anti-apoptotic properties of the IAPs, because they are involved in binding and reversible inhibition of specific caspases [183-185].

Regulation of the anti-apoptotic function exerted by IAPs can be achieved by tight transcriptional control on one hand and post-transcriptional control on the other hand. IAP expression is regulated by the stress responsive transcription factor nuclear factor of κB (NF-κB) [186]. In most cells NF-κB activation protects against apoptosis through the induction of survival genes. Beside IAP, these genes code for anti-apoptotic Bcl-2-family members and several signaling decoys [187-190].

Furthermore, many IAPs harbor a C-terminal zinc-binding motif known as RING domain [191] that is implicated in the process of ubiquitin conjugation. The RING domain of several IAPs, namely X-linked IAP (XIAP), c-IAP1 and c-IAP2, show ubiquitin-ligase activity [192]. IAPs are involved in mediating the ubiquitination of caspases, thus preventing the initiation of a caspase cascade [193,194]. In addition, the ubiquitin-ligase domain has been shown to promote auto-ubiquitination and subsequent degradation of IAPs [192,194]. IAP stability is regulated by ubiquitination through the association with specific pro-apoptotic proteins [Figure 4 and ref. 192].

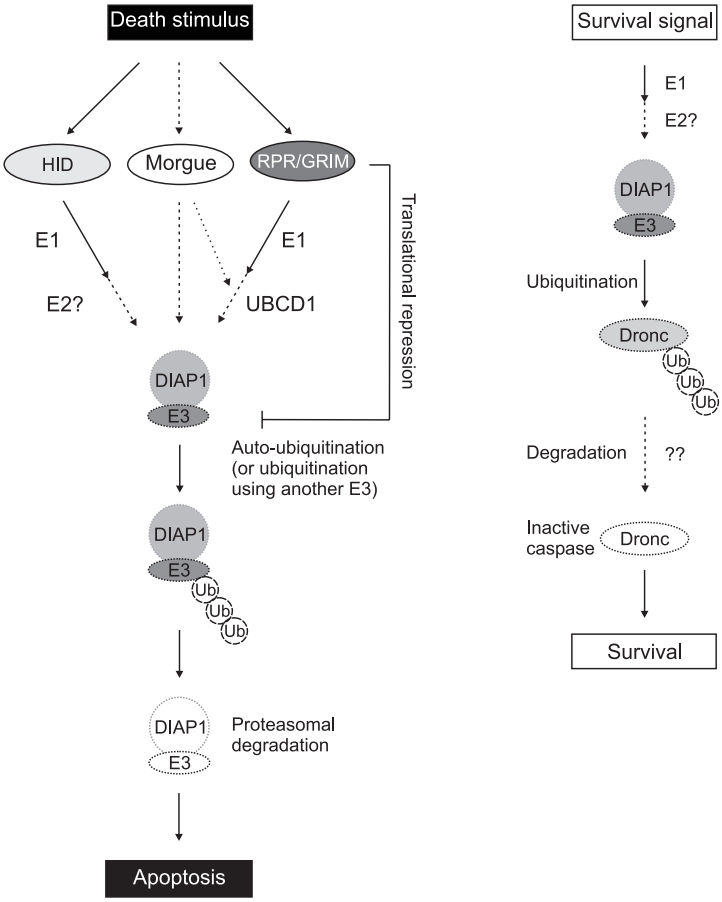



**Figure 4. Regulation of apoptosis through DIAP1.** After apoptotic stimuli, DIAP1 is either degraded through Reaper, Grim, Hid or Morgue via ubiquitin-dependent proteolysis. Furthermore, protein levels can drop due to translational repression. In the absence of an apoptotic stimulus, DIAP1 mediates the ubiquitination of DRONC, which, in turn, prevents the activation of downstream caspases, resulting in cell survival. Dotted lines indicate possible, but to date unproven pathways.

### *Inhibitors of Inhibitor of Apoptosis Proteins*

In 2000 the first mammalian IAP antagonist was identified, referred to as direct IAP binding protein with low pI (Diablo) or second mitochondria-derived activator of caspases (Smac) [195,196]. Diablo/Smac is produced as a precursor with a mitochondrial import sequence at its N-terminus that is removed upon import into the inter-membrane space of mitochondria [195,196]. This IAP antagonist resides within mitochondria in healthy cells and, upon cellular stress, Diablo/Smac is released into the cytosol to promote cell death by preventing IAP inhibition of caspases by interacting with IAP's BIR motifs [197,198]. Like cytochrome c, release of Diablo/Smac from mitochondria is regulated by Bcl-2 family members [195,199]. The four N-terminal residues of

Diablo/Smac (AVPI) are essential for interacting with a surface groove of BIR3 of XIAP and for the activation of apoptosome [200,201].

This conserved motif is also found in *Drosophila* proteins Hid, Grim and Reaper. These proteins interact with the *Drosophila* IAP (DIAP1) and behave as the functional homologues of Diablo/Smac by antagonizing the ability of DIAP1 to bind to the *Drosophila* caspase Dronc [197,202,203]. Therefore, the N-terminal peptide is also known as Reaper/Hid/Grim (RHG) motif. The mechanism used by this domain is thought to be conserved during evolution. This is supported by the fact that several *Drosophila* RHG-containing proteins can bind XIAP, and decreases the ability of XIAP to bind caspase-9 [204]. Reaper, Grim and Hid can all promote the degradation of DIAP1 through ubiquitination. However, in some instances it is apparent that Reaper and Grim are critical for ubiquitination of DIAP1, whereas in other situations Hid initiates this ubiquitination [Figure 4 and ref. 204-207].

Reaper and UBCD1, a *Drosophila* ubiquitin-carrier enzyme-like protein, bind to DIAP1 and promote auto-ubiquitination of DIAP1 [Figure 4 and ref. 205]. Already many years ago, when increased ubiquitin levels were detected in dying cells of the intersegmental muscles of the *Manduca Sexta*, it was suggested that ubiquitin-mediated proteolysis must play an important role in programmed cell death [208]. Further evidence came in 1996 from two studies showing that inhibition of the proteasome leads to the inhibition of apoptosis in thymocytes as well as in neurons [209,210]. In addition, Reaper and Grim regulate DIAP1 levels by a general inhibition of translation that is induced by a yet unknown process [204]. Although Reaper can participate in both ubiquitin-dependent degradation and translation inhibition, different regions of the Reaper protein mediate these distinct processes [204].

Whereas mammalian Diablo/Smac is constitutively expressed in many healthy cells and its action is controlled by its localization within mitochondria, the *Drosophila* IAP antagonists Reaper, Grim and Hid are regulated at the level of transcription [211]. Several death signals transcriptionally activate Reaper expression [212]. Reaper levels decline rapidly when expressed individually, whereas co-expressions of IAPs inhibit this decline. In the presence of IAPs the cytosolic localization of Reaper was changed into a punctate perinuclear localization that resembles IAP localization. Recently, another study has identified a domain in Grim that is required for targeting Grim to mitochondria. This domain, termed GH3 domain, can activate a pro-apoptotic pathway distinct from IAP inhibition, which is promoted by the N-terminal domain [213]. The GH3 domain shows sequence homology with regions in Reaper and Sickle, a recently identified *Drosophila*

RHG domain containing protein [206,213]. This sequence homology suggests function conservation of the domain between these three pro-apoptotic proteins.

Recently, Morgue, an enhancer of Reaper/Grim induced apoptosis, was identified [206]. Morgue is a unique protein that encodes both a ubiquitin-conjugase domain and an F-box domain, a motif found in many ubiquitin ligases and involved in regulating ubiquitination. Morgue regulates DIAP1 through ubiquitination and degradation. Reaper and Grim can synergize with Morgue in the degradation of DIAP1. Possibly, Morgue functions as a ubiquitin conjugase and through interaction with DIAP1 promotes its auto-ubiquitination [214]. Furthermore, a negative regulator of the apoptotic effect of Reaper has been identified, termed Scythe [215] (see section 'GHR INTERACTING PROTEINS' p. 44).

## Anti-Apoptotic and Proliferative Actions of GH

Many hormones, cytokines, and growth factors have been shown to be regulators of cell death. Steroid hormones can act as survival factors in the mammary gland, prostate, ovary, and testis [216]. Epidermal growth factor, platelet derived growth factor, nerve growth factor and insulin-like growth factor (IGF-I) are able to inhibit apoptosis in hemapoietic cells and neurons [217].

Recently, evidence accumulated that GH can also act as an inhibitor of apoptosis. A protective action of GH on Fas-mediated apoptosis in U937 monocytes can be observed due to enhanced expression of the anti-apoptotic oncoprotein Bcl-2 as well as increased levels of *bcl-2* mRNA [218]. During *in-vitro* embryogenesis GH acts as an effective inhibitor of apoptosis. Expression of Bcl-2 has not been affected by GH treatment whereas synthesis of Bax has been reduced, thereby altering the Bax to Bcl-2 ratio [219]. In addition, Jeay *et al.* have shown that GH can act as a survival factor in the pro B Ba/F3 cell line transfected with rat GHR (Ba/F3 GHR) [Figure 5 and ref. 220,221]. This mitogenic effect of GH is not exerted by IGF-I because IGF-I mRNA is not transcribed in Ba/F3 GHR cells [222]. As cells are able to produce small amounts of GH, an autocrine/paracrine way of GH signaling is anticipated. This signaling pathway results in the activation of nuclear factor-κB (NF-κB) that leads to the sustained expression of Bcl-2 and potentially BAG-1 [221]. NF-κB can regulate among others the expression of proteins involved in cell survival, namely Bcl-2, Bcl-$x_L$ [187,223], c-IAP-1, c-IAP-2 [224], c-myc [225] and cyclin D1 [226].

In a human leukemia cell line (HL-60 cells) Costoya *et al.* have shown that GH activates Akt in a PI-3-kinase dependent manner [227]. In Ba/F3 GHR cells increased concentration of GH promote proliferation that depends on the activation of PI-3-kinase,

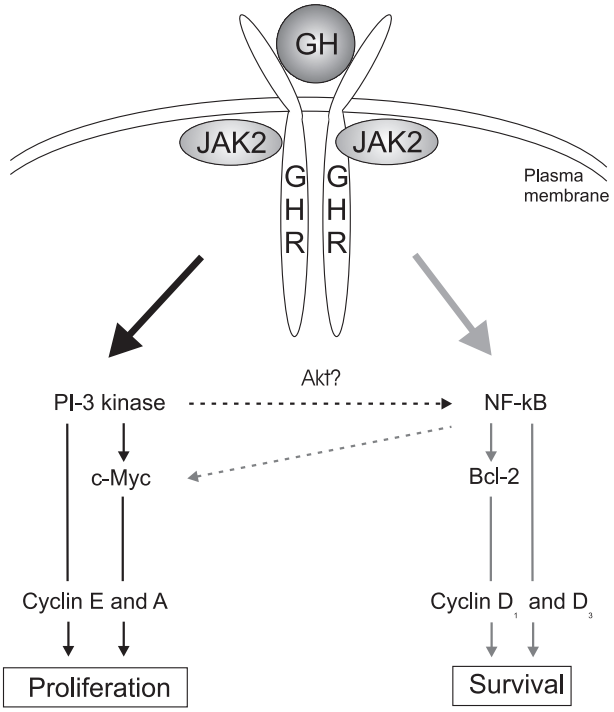



**Figure 5. Schematic representation of signaling pathways involved in suvival and proliferative signaling induced by GH.**
GH binding to its receptor promotes JAK2 activation, which induces - among others - activation of PI-3-kinase (black arrows) and NF-kB (gray arrows) signaling pathways. PI-3-kinase activation accounts for the proliferative response, whereas NF-kB activation is responsible for cell survival. A link between these two pathways might exist (indicated by dotted lines). This figure has been adapted from ref. [222].

leading to the activation of the proto-oncogene c-*myc* [220,228]. Akt, a serine/threonine kinase, has been implicated in the regulation of survival mechanisms in several cell types [229]. Activated Akt phosphorylates and thereby inactivates a number of targets, including pro-apoptotic Bad and caspase-9. Furthermore, it is believed that PI-3-kinase regulates NF-κB activation via activation of downstream Akt [230].

Finally, the cell cycle regulation by cytokines is closely related by their capacity to express cyclins [231]. Growth factor deprivation reduces cyclin synthesis and promotes cell cycle arrest, which can lead to apoptosis. In Ba/F3 GHR cells GH-mediated induction of proliferation correlates with the induction of cyclins E and A and c-myc expression by a PI-3-kinase dependent pathway. At the same time, the expression of $p27^{kip1}$, a cycline-dependent kinase inhibitor that inactivates cyclins [231], is repressed [222]. On the other hand, under starvation conditions endogenous levels of GH sustain activation of NF-κB in Ba/F3 GHR cells, which leads to maintained levels of cyclin D1 and D3 and cell survival [220].

In Ba/F3 cells, the anti-apoptotic effect of GH is primarily associated with activation of NF-κB, as cell survival cannot be restored by GH in the presence of a

dominant-negative NF-κB [221]. In addition, inhibition of PI-3-kinase in the presence of GH leads to growth arrest without any mortality, indicating that only a part of PI-3-kinase is involved in the activation of NF-κB induced by GH [220]. The PI-3-kinase pathway accounts for the proliferative GH response, probably through c-myc activation and/or expression [220].

Elevated levels of GH, as seen in acromegalic patients, can be expected to lead to unintentional signaling of cell survival. This is indeed the case, as acromegaly is related to the development of acute promyelocytic leukemias and non-Hodgkin's lymphomas, which both produce high GH levels [232-234]. One can speculate that the overexpression of GH could lead to constitutive activation of NF-κB. Indeed, there is evidence that links constitutive activation of NF-κB to the development and the progression of a wide range of cancer, including Hodgkin's lymphomas [235], B cell lymphoma and T cell leukemias [236,237].

# Ubiquitin-Proteasome System

The evolutionary-conserved 76 amino acid polypetide ubiquitin accomplishes essential functions in eukariotes through its covalent conjugation to other intracellular proteins [238]. Initially identified as a protein that marks abnormal or misfolded proteins for degradation by the proteasome, it now becomes apparent that ubiquitin is involved in a variety of cellular processes, which do not necessarily engage degradation. Ubiquitination has been implicated in a variety of basic pathways during cell life and death, such as progression of cell cycle, induction of inflammatory response and antigen presentation, postreplicational DNA repair, function of certain transcription factors, immune response, protein quality control, protein transport, and apoptosis. Abnormalities in ubiquitin-mediated processes can cause pathological conditions, including malignant transformation and several inherited diseases [239].

## Ubiquitin Conjugation

Usually, ubiquitination results in the formation of a bond between the C-terminus of ubiquitin (G76) and the ε-amino group of a substrate lysine residue. In some cases the α-amino group of the N-terminal residue in the substrate can be used for ubiquitin conjugation [240-242]. Ubiquitination requires the successive action of three enzymes [Figure 6 and ref. 243]. Initially, in an ATP-dependent reaction, the ubiquitin-activating enzyme E1 forms a thiolester with the carboxy group of the C-terminal glycine residue

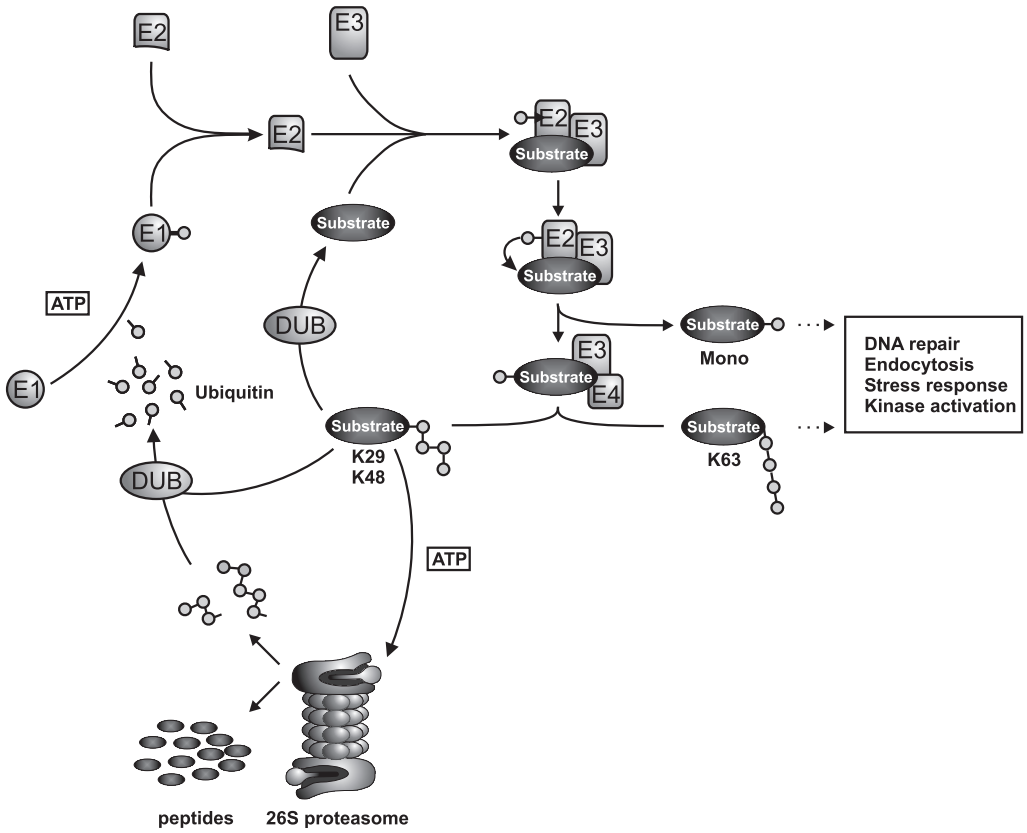



**Figure 6. Schematic representation of the ubiquitin-proteasome pathway.**
Ubiquitin is conjugated to substrate molecules by the successive action of E1, E2, and E3. In some cases, a ubiquitin-chain elongation factor, E4, is required to drive poly-ubiquitin chain assembly. Poly-ubiquitin chains linked through K29 or K48 are targeting signals for degradation through the 26S proteasome. Mono-ubiquitination can signal endocytosis of some substrates. K63-linked ubiquitin chains function in DNA repair, stress response, kinase activation or endocytosis. De-ubiquitinating enzymes (DUB) can remove ubiquitin from ubiquitinated substrates or release free ubiquitin from poly-ubiquitin precursors as well as poly-ubiquitin chains released by the proteasome and restore the cellular ubiquitin pool.

of ubiquitin. One of several ubiquitin-carrier enzymes (E2) receives the activated ubiquitin from E1, forming an additional high-energy thiolester intermediate, E2-S~ubiquitin. Finally, the last step in the conjugation process can be mediated directly by the E2, but frequently requires a ubiquitin-protein ligase (E3). This three-step mechanism initiates all known ubiquitination reactions, independent of whether ubiquitin signals degradation, endocytosis, or some other fate.

Number and topology of the substrate-conjugated ubiquitins are important signals for the possible consequences of ubiquitination [244]. A single ubiquitin can be linked to a substrate (mono-ubiquitination), but also to a lysine of a ubiquitin moiety that is already linked to the substrate resulting in poly-ubiquitin chains. Recently, a ubiquitin-chain elongation factor (E4) has been identified that is involved in the extension of short poly-ubiquitin chains [245]. If these poly-ubiquitin chains are linked via lysine 29 or lysine 48 of ubiquitin, the substrates are efficiently targeted for degradation by a large multimeric cytosolic protease, the 26S proteasome [244]. Unlike the substrate, the ubiquitin molecules are recycled for new conjugation reactions. De-ubiquitinating enzymes (DUBs) cleave ubiquitin from proteins and disassemble multi-ubiquitin chains in order to sustain a sufficient level of free ubiquitin [246]. Ubiquitin chains linked through lysine 63 have been implicated in non-proteolytic signaling such as regulation of endocytosis of membrane proteins [247], DNA repair [247,248], and kinase activation [249].

### *Ubiquitin-activating enzyme E1*

In most organisms, including humans and yeast *Saccharomyces cerevisiae*, a single E1 enzyme activates ubiquitin for the entire array of downstream carrier enzymes. Alternative translation starts in mammalian cells yields two isoforms of 110 kDa and 117 kDa, respectively [250]. E1 is an essential enzyme, and cell lines expressing a temperature-sensitive E1 undergo cell cycle arrest at non-permissive temperature [251].

### *Ubiquitin-carrier enzyme E2*

In yeast *Saccharomyces cerevisiae* eleven E2s have been identified and even more in higher organisms, reflecting multiple isoforms of some E2s [252]. All known E2s share a conserved core domain of approximately 150 amino acids, termed UBC domain. Within this UBC domain lies the conserved cysteine residue required for ubiquitin thiolester linkage [253]. Certain family members feature N- or C-terminal extension that may facilitate specific interactions with E3s or may serve as membrane anchor and contribute to the cellular localization [254,255]. Typically, each E2 interacts with a number of E3s, thus being involved in targeting numerous substrates.

### *Ubiquitin-protein ligase E3*

The E3 reaction involves at least two distinct steps. First, an E3 binds its substrate via the ubiquitination signal and interacts with an E2. Second, the E3 is involved in the

covalent ligation of one or more ubiquitins to the substrate or the extension of ubiquitin chains onto substrate-linked ubiquitins. Since the E3s bind directly or via ancillary proteins to their substrate, E3s are the key players in determining the specificity of the system. All known E3s feature one of three structural elements, namely a HECT domain, a RING finger or a U-box.

**HECT E3 ligases**

HECT stands for Homologous to E6-AP C-terminus. E6-AP is the founder of this E3 family, which mediates the degradation of the tumor suppressor p53 in the presence of the human papilomavirus oncoprotein E6 [256]. The HECT domain is a region of 350 amino acids [257]. This region bears a conserved cysteine residue, to which the activated ubiquitin moiety is transferred from the E2 [258]. This cysteine of E6-AP is required for p53 ubiquitination because it acts as a site of thiolester formation with ubiquitin. Experimental evidence indicates that all HECT domain E3s use a similar mechanism of covalent catalysis [259], often with UbcH7 or UbcH8 as the cognate E2 [260-262]. The unique N-terminus of each family member interacts with specific substrates. A feature that is shared by many HECT E3s is the presence of a WW domain, which interacts with proline rich PY motifs of the substrate [263]. In addition, most HECT domain E3s possess an N-terminal C2 domain that mediates translocation to the plasma membrane in response to increased intracellular $Ca^{2+}$ levels.

One of the best-characterized human HECT E3 is neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4). Nedd4 mediates the ubiquitination of the epithelial sodium channel (ENaC). The ubiquitination of ENaC requires the localization of Nedd4 to the membranes via its C2 domain [264] and relies on the recognition of PPXY motifs in ENaC by WW domains of Nedd4 [265]. The inherited disorder known as Liddle syndrome involves mutations of these PPXY motifs, which prevent ENaC ubiquitination. Liddle syndrome causes hypertension due to the retention of the sodium channel at the plasma membrane [265,266]. The yeast homologue of Nedd4, Npi1/Rsp5, regulates a set of plasma membrane permeases and receptors [247]. For these substrates, the consequence of ubiquitination is believed to be endocytosis rather than proteasomal degradation.

**RING E3 ligases**

Members of the really interesting new gene (RING) E3s are believed to mediate the direct transfer of ubiquitin from E2 to the substrate [191]. RING finger domains are defined by a pattern of conserved cysteine and histidine residues that bind two zinc ions [267,268]. RING fingers function not as chemical catalysts but as molecular scaffolds

that bring substrate and E2 in close proximity [269]. RING E3s come in two varieties: the single subunit and the multi-subunit RING E3s [270].

The single subunit RING E3s contain both the E2- and the substrate-binding site on the same molecule, as for example in c-Cbl, which is involved in the ubiquitination of growth factor receptors [271]. Other representatives of this type of E3 are Mdm2, which ubiquitinates the tumor suppressor protein p53 [272], and the inhibitor of apoptosis (IAP), which are implicated in the down-regulation of caspases under non-apoptotic conditions [193,194].

In multi-subunit RING E3s interaction with E2 and the substrate is mediated via separate subunits of the multi-protein complex. Among them is the anaphase promoting complex (APC) or cyclosome involved in the degradation of cell cycle regulators [273], the Skp1-Cullin1/Cdc53-F-box protein SCF-RING finger complex that mediates the degradation of signal- and cell cycle-induced phosphorylated proteins [274], and the von Hippel-Lindau/Elongin C/Elongin B/Cullin2 (VHL-CBC) complex, a regulator of hypoxia-inducible transcription factor 1α (HIF1α) [275]. The organization of the SCF E3s and VCB E3s is very similar. In each case, an approximately 100-residue RING finger protein known as Rbx1/Hrt1/Roc1 plays a key organizational role. Rbx1 interacts strongly with a subunit belonging to the Cullin protein family (Cul1/Cdc53 in SCF E3s, Cul2 in VCB). In addition, Rbx1 is involved in the recruitment of the cognate E2. In multi-subunit RING E3s substrate recognition is delegated to a separate subunit. In the SCF E3s, substrate-specific F-box proteins are recruited to SCF complexes through the adaptor protein Skp1, which recognizes the F-box motif [274]. The substrate-recognition subunit of VCB is pVHL, the product of the Von Hippel-Lindau tumor suppressor gene [276]. pVHL is recruited to the complex through interactions with the heterodimeric adaptor Elongin B/C. Many cancer associated pVHL mutations are predicted to destabilize the interaction of pVHL with Elongin B/C [277]. Many of the numerous F-box proteins in the databases represent the substrate recognition components of a large family of SCF E3s (for example Skp2, Cdc4, Grr1 or the transducing repeat-containing protein β-TrCP). Similarly, some of the numerous proteins that share an Elongin B/C interaction motif are the members of the supressors of cytokine signaling (SOCS), which may be the substrate specificity components of a family of VCB-like E3s [277]. Therefore, these multimeric RING E3s are probably involved in the regulation of cytokine receptor signaling [141]. Interestingly, both Cullin1 and Cullin2 can be modified with the ubiquitin-like protein Rub1 (mammalian Nedd8), which increases the E3 ligase activity of the complex [278].

**U-box E3 ligases**

Recent studies indicate that U-box containing proteins form a third class of proteins that function as E3 enzymes [279]. The U-box is a 75 amino acid motif that has been suggested to adopt a tertiary structure very similar to that of a RING domain, with the exception of lacking the zinc-binding motif and utilizing salt bridges to stabilize its structure [280]. Nevertheless, U-box proteins are still capable of binding Ubc4/5 E2 [281]. The first identified U-box protein is the yeast ubiquitination factor ubiquitin-fusion degradation protein 2 (UFD2). UFD2 has been assigned an E4 activity that regulates the number of ubiquitin residues attached to a model substrate [282]. UFD2 appears to participate in generating ubiquitin chains by making specific ubiquitin linkages [283]. UFD2 may complete the job of another ubiquitin ligase that does not add ubiquitin chains of sufficient length or linkage to trigger degradation. Such a situation may be particularly useful if short or atypical ubiquitin chains provide an alternative signal other than to promote degradation. More insight in the substrates of UFD2 may shed light on the relation of E3 and E4 activity.

C-terminus of Hsc70/Hsp70 interacting protein (CHIP), the best characterized U-box protein, has initially been identified as a tetratricopeptide repeat-containing protein (TPR). CHIP uses this protein-protein interaction domain to bind to the cytoplasmic

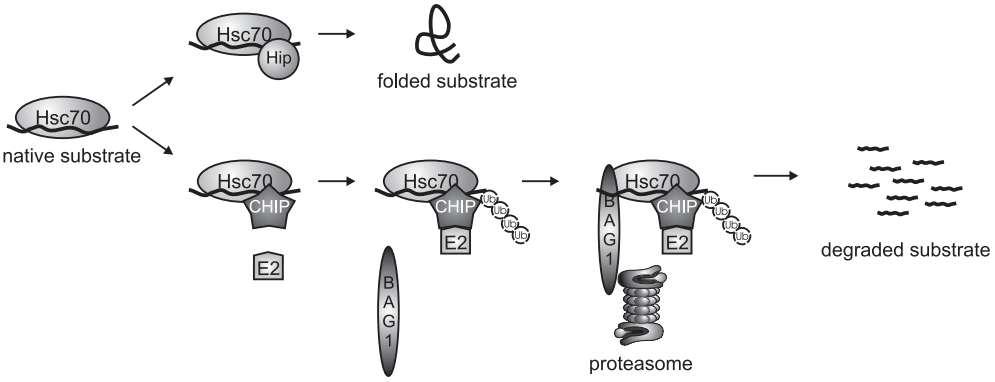



**Figure 7. Involvement of CHIP, Hsc70/Hsp70, and BAG-1 in protein degradation.**
A native substrate can be correctly folded by Hsp70/Hsc70 together with a 'productive' co-chaperone, like Hip. However, if Hsc70/Hsp70 acts in concert with TPR co-chaperones, such as CHIP, the substrate enters the degradative pathway. CHIP can recruit an E2 ubiquitin-carrier enzyme of the Ubc4/5 family to the chaperone complex and mediate ubiquitination of the substrate in conjunction with the E2. The ubiquitinated substrate is then targeted for degradation to the proteasome, which can be linked to the complex by BAG-1. BAG-1 binds to Hsp70/Hsc70 and utilizes its ubiquitin-like domain for association with the proteasomal.

molecular chaperones, Hsc70 and Hsp90 [284]. A U-box domain is present within the C-terminus of CHIP, and recent data show that CHIP can function as a ubiquitination factor for chaperone bound substrates, like the cystic fibrosis transmembrane conductance receptor or the glucocorticoid receptor [285,286]. In the case of these proteins, CHIP removes them from the folding pathway and diverts them to the ubiquitin-proteasome pathway for degradation (Figure 7). This diversion results from the ability to inhibit chaperone function and from ubiquitin-ligase activity of CHIP. The capability to redirect proteins from the productive folding pathway may be an important function of the cell in removing proteins that are too damaged to be folded and do not function properly. It is still not clear what triggers the switch from protein folding to protein degradation [287], but CHIP could participate in this process because of its capacity to interact with components of the chaperone machinery and the proteasome system [285]. Ubiquitination of chaperone substrates and their targeting for degradation through the proteasome appears to be a highly regulated process. Another co-chaperone of Hsc70, the anti-apoptotic protein BAG-1, interacts physically and functionally with CHIP to assist in transferring ubiquitinated chaperone substrates to the proteasome [288]. Recently, Imai *et al.* reported that CHIP and Hsp70 are binding partners of Parkin, a RING E3 [289,290]. Mutations in Parkin are associated with an autosomal recessive form of Parkinson disease [291]. The substrate of Parkin is the unfolded Peal receptor (Peal-R) [292], which transiently associates with Hsp70 and thereby inhibits the E3 activity of Parkin. CHIP enhances the E3 activity of Parkin by promoting the dissociation of Hsp70 from the Parkin-Peal-R complexes [289]. This is another example in which CHIP promotes the degradation of an unfolded or poorly folded protein, suggesting a general role of CHIP as a negative regulator of chaperone function that is responsible for the decision to degrade a misfolded protein.

## Proteasome

The proteasome is the predominant protease in the cytosol that accounts for 1% of all cellular proteins [293]. The 26S proteasome is a large cylinder-shaped complex composed of a 20S catalytic core particle that contains the protease subunits and the 19S regulatory particle that, in turn, regulates the function of the 20S particle [294,295]. One or two regulatory particles attach to each end of the 20S particle to form the 26S proteasome.

The 20S particle is a hollow cylindrical structure composed of four stacked rings: two identical outer $\alpha$ rings and two identical inner $\beta$ rings. Each of the two outer rings is

comprised of seven genetically related and structurally similar α-subunits, and each of the inner rings is comprised of similarly conserved β-subunits. Three β-subunits contain proteolytic activity and hydrolyze proteins into peptides of about eight amino acids. These peptides can be further hydrolyzed to amino acids by cytosolic peptidases [296] or, alternatively, be presented to the immune system by major histocompatibility complex (MHC) molecules [295].

The 19S particle can be further dissected into two multi-subunit substructures, a lid and a base [297]. Six homologous ATPases (Rpt1-6) are present in the base together with three non-ATPase subunits (Rpn-1, Rpn-2, and Rpn-10). Direct interactions have been reported between these ATPases and the α-subunits of the 20S particle [298]. The ATPases in the base most likely unfold substrates and translocate them through a gated channel into the 20S particle [299]. The role of the lid is still unclear, although it is necessary for proper degradation of poly-ubiquitinated proteins [297]. One of the functions of the 19S complex is the recognition of ubiquitinated substrates, which can be mediated via the Rpn-10 subunit (referred to as S5α in humans) [300]. However, since this subunit is not essential in yeast, additional ubiquitin-binding components must exist [301].

Usually, proteasomes completely degrade substrates into small peptides, but in a few cases, degradation can yield biologically active protein fragments like in the case of two yeast transcription factors, Spt23 and Mga2. Both proteins are synthesized as inactive precursors that are anchored to the endoplasmatic reticulum through their C-terminal tail. Their activation depends on regulated ubiquitin-proteasome dependent processing (RUP) [302]. In case of the transcription factor NF-κB limited processing of the precursor protein p105 in p50 by the proteasome has been reported [303]. Proteasomal processing occurs after ubiquitination of p105, and is stopped by a glycine-rich repeat that prevents the degradation of p50 [304]. Strikingly, a glycine-alanine repeat precludes any proteasomal degradation of the Epstein-Barr virus nuclear antigen 1 (EBNA1) [305]. This prevents the presentation of EBNA1 anigenic peptides by MHC molecules, and consequently EBNA1 escapes the immune system. So far, ubiquitination was thought to be required for targeting a substrate to degradation by the proteasome. However, ubiquitin-independent targeting has been reported in the case of ornithine decarboxylase and $p21^{cip1}$ [306,307].

## Regulation of Membrane Proteins

It has been common knowledge that the ubiquitin system is involved in selective

degradation of cytosolic and nuclear proteins. However, presently it is generally accepted that the system is also involved in the degradation of membrane proteins, as well as in the trafficking of a variety of membrane proteins at several intracellular locations [308-311].

### *Endoplasmic Reticulum*

Protein degradation in the ER is essential for the elimination of misfolded proteins from the secretory pathway and is mediated by the cytosolic ubiquitin-proteasome machinery [312,313]. Misfolded proteins are recognized by ER chaperones such as calnexin and Bip (Kar2 in yeast), and trigger the 'unfolded protein response' (UPR) [314]. Translocation to the cytosol via the Sec61 translocon and poly-ubiquitination finally result in the degradation by the proteasome in the cytosol. This quality control mechanism is referred to as ER associated degradation (ERAD) and ensures that only correctly folded and/or assembled proteins reach their destination in the cell. Among membrane proteins degraded via ERAD are normal and mutant cystic fibrosis transmembrane conductance regulator (CFTR) [315] and yeast and human 3-hydroxy-3-methyl-coenzyme A reductase (HMGR) [316].

### *Trans-Golgi Network*

Recently, a role of ubiquitination at the *trans*-Golgi network (TGN) in the routing of two yeast proteins was observed. The yeast general amino acid permease 1 (GAP1) [317,318] and the tryptophan permease TAT2 are ubiquitinated depending on nutrient conditions and are subsequently directed from the TGN to the vacuole (the yeast equivalent of the mammalian lysosome) without reaching the plasma membrane. Lysine 63 linked ubiquitination is probably responsible for this sorting event, without a role for the proteasome [319]. This down-regulation requires ubiquitination of GAP1 through a process involving ubiquitin ligase Rsp5, ubiquitin hydrolase Doa4, and Bul1/2, two Rsp5 interacting proteins [320].

### *Plasma Membrane*

Numerous cell surface receptors, permeases and transporters undergo ubiquitination on specific lysine residues. Ubiquitination or action of the ubiquitin system is believed to be required for the first step in endocytosis, namely the entry into vesicles budding from the plasma membrane. In yeast constitutive ubiquitination, ubiquitination in response to ligand binding or changes in growth conditions regulate the internalization

of – among others – the α-factor (Ste2p) and a-factor receptor (Ste3p), permeases of uracil (Fur4p), maltose (Mal61p) and general amino acids (Gap1p), as well as ABC-transporter Ste6p [321]. In the mammalian cells several signal-transducing receptors and ion channels, like the GHR, the EGF receptor, the PDGF receptor, Met receptor, and ENaC, are ubiquitinated [322]. In most cases, the proteasome does not recognize these ubiquitinated proteins; instead, the ubiquitinated plasma membrane proteins are targeted for degradation in the lysosome/vacuole. In yeast mono-ubiquitination appears to be sufficient to trigger endocytosis, because in frame fusion of ubiquitin to Ste2p or the otherwise stable plasma membrane protein Pam1 stimulates endocytosis [323,324]. For several yeast amino acid permeases, di-ubiquitin chains linked through lysine 63 further enhance internalization rates [325,326]. Ubiquitination probably functions in activating the internalization and endocytic machinery. Ubiquitin lacks classical internalization motifs, like the di-leucine motif or the tyrosine-based internalization motif, which might serve as docking sites for the endocytotic machinery [327]. However, there is evidence that the three dimensional structure of ubiquitin carries the internalization signal that can be appended to the plasma membrane protein to trigger internalization. This internalization signal is composed of two surface patches surrounding the critical amino acid residues phenylalanine 4 and isoleucine 44 [328]. But ubiquitin moieties alone are not enough for internalization. The internalization of a yeast receptor-ubiquitin chimera that does not need to be ubiquitinated prior to endocytosis, still requires ubiquitin-carrier enzymes and the Rsp5 ubiquitin ligase [329].

In mammalian cells the uptake of the activated G protein coupled $\beta_2$-adrenergic receptor ($\beta_2$AR) depends on the RING E3 Mdm2 [330]. Activation of $\beta_2$AR results in the recruitment of β-arrestin, an adaptor that can interact with both clathrin and clathrin adaptor proteins 2 (AP-2) [331]. At the same time ubiquitination of β-arrestin and $\beta_2$AR takes place, whereby the ubiquitination of β-arrestin is required for endocytosis by clathrin-coated pits. Furthermore, in cells lacking Mdm2, ubiquitination of β-arrestin (but not the receptor) is lost, resulting in decreased receptor internalization, but with little effect on receptor degradation [330]. It is likely that the ubiquitin moieties on β-arrestin mediate the linkage to the endocytic machinery.

Furthermore, compelling evidence accumulates that ubiquitin is linked to the endocytotic machinery by proteins that contain a ubiquitin-interacting motif (UIM). This motif has originally been identified in S5α subunit of the 19S regulatory particle of the proteasome, which has a preference for chains containing four or more ubiquitin monomers. Interestingly, UIMs are also present in Eps15, Eps15R, and epsin, which can

all interact with components of the endocytotic machinery, including clathrin and the AP-2 complex [332,333]. Thereby, UIM containing proteins might be involved in binding to ubiquitinated cell surface proteins, which lack a classical internalization motif, and recruit these ubiquitinated proteins into membrane pits that are coated with the clathrin protein complex [334].

### *Endosomes*

Cell surface receptors that have been internalized and delivered to the endosomal system can either be recycled to the plasma membrane or degraded via delivery to the lumen of the lysosome/vacuole [335]. At the endosomal membrane, ubiquitin again directs the sorting of receptors, but here they are recruited into 60 nm vesicles that bud away from the cytosol into the lumen of the endosome [336]. As the endosome matures it accumulates internal vesicles and takes on the characteristic appearance of the multivesicular body (MVB). Fusion of the limiting membrane of the MVB with that of the lysosome exposes the vesicles to lysosomal lipases and proteases that degrade both the vesicles and their contents [337].

The ubiquitin tag is used by a variety of cell surface transporters and permeases, as well as proteins of the biosynthetic pathway, such as yeast vacuolar proteases, that are sorted into the internal vesicles of the MVB for subsequent delivery to the lysosome/vacuole [338]. One of the best-characterized cell surface proteins sorted in the MVB pathway is the activated EGFR. Upon binding of EGF, activated EGFR is rapidly endocytosed, ubiquitinated by c-Cbl, a RING E3, internalized into MVB and degraded following delivery to the lumen of the lysosome [339,340]. Oncogenic forms of c-Cbl that are defective for ligase activity result in the recycling of the activated EGFR back to the plasma membrane and prolonged EGFR signaling, which is thought to contribute to tumorigenesis [341-343]. The sorting of yeast vacuolar hydrolase carboxypeptidase S (CPS) to the vacuole via the MVB pathway depends on the mono-ubiquitination of a single lysine [339].

The cargo selection machinery that recognizes ubiquitinated proteins has recently been identified in yeast as the endosomal sorting complex required for transport-I (ESCRT-I) [339]. ESCRT-I is a 350kDa protein complex consisting of the class E vacuolar protein sorting (Vps) proteins Vps23, Vps28, and Vps37. Vps23 and its mammalian homologue tumor susceptibility gene 101 (Tsg101) contain a UBC-like domain that resembles the UBC domain of E2 enzymes but lacks the active site cysteine [344]. Nevertheless, Vps23 is capable of binding ubiquitinated substrates through its

UBC-like domain and sort them into internal vesicles of the MVB [339]. Mutation of this domain prevents both sorting and formation of vesicles suggesting that the recognition of ESCRT-I activates downstream components of the vesicle budding machinery [345].

Two other proteins required for the transport to the vacuole strengthen the concept that ubiquitin is involved in the cargo selection for MVB internal vesicles: (1) Vps27, an ortholog of mammalian Hrs, has a UIM that mediates the binding of ubiquitin [346,347]. Furthermore, Vps27/Hrs, like epsin, possesses a specific phosphoinositide (PI)-binding domain immediately upstream of the UIM. Different forms of PIs are found in different cellular membranes and could therefore result in the recruitment of different UIM containing proteins to distinct membranes by specific lipid binding domains and act by binding ubiquitinated cargo and assembling members of the budding machinery [347]. There are indications that Vps27 may function upstream of ESCRT-I in cargo selection [348]. (2) Doa4, a de-ubiquitinating enzyme, removes ubiquitin from proteins targeted for degradation before internalization of the targeted proteins into the vacuole [349]. This allows the recycling of the ubiquitin moiety, making it available for new conjugation reactions.

Recently, Emr and coworkers have described two novel ESCRT complexes, ESCRT-II and ESCRT-III, that act together with ESCRT-I to regulate MVB sorting [350,351]. ESCRT-II is a soluble, stable 155 kDa protein complex formed by the class E Vps proteins Vps22, Vps25, and Vps36. In contrast, ESCRT-III consists of soluble coiled-coil-containing proteins Vps2, Vps20, Vps24, and Snf7, which are recruited from the cytosol to the endosomal membranes. Here they oligomerize into the ESCRT-III protein complex. The current data of the sorting event at the MVB suggest the following model: ESCRT-I binds to ubiquitinated cargo, which leads to the transient recruitment of ESCRT-II to the endosomal membrane. ESCRT-II recruits and assembles the components of the ESCRT-III complex. The combined action of the three ESCRT complexes results in the concentration of cargo into membrane invaginations that bud to form vesicles in the lumen of MVB. After budding is completed, the ESCRTs are released from the membrane by the action of the Vps4 AAA-ATPase.

In summary, ubiquitin is a posttranslational modification that functions in diverse sorting/transport steps of membrane proteins in both the biosynthetic and the endocytic pathway. Single ubiquitin moieties attached to the cargo or alternatively linked poly-ubiquitin chains are able to function as a specific sorting signal. In concert with cargo recruiting E3s that can function at diverse locations of the cell and result in mono- or specific poly-ubiquitination, an explicit sorting mechanism is available in the cell based

on a single molecule: ubiquitin.

## Regulation of the GHR

The GHR was initially found ubiquitinated by amino acid sequencing of purified receptor from the liver [65]. Subsequently, evidence accumulated that the ubiquitin system is necessary for ligand-induced internalization and degradation of the GHR [352]. By expressing the GHR in a Chinese hamster ts20 cell line, which harbors a temperature-sensitive E1 enzyme, non-ubiquitinated GHR accumulated at the cell surface, whereas ubiquitination of the GHR was enhanced after ligand binding at the permissive temperature. GHR ubiquitination occurs at the plasma membrane and coincides with the recruitment of the receptor into clathrin-coated pits [353]. Ubiquitination of the GHR itself is not required for internalization. Internalization of a GHR (1-399) truncation mutant, in which all ten intracellular lysine residues are substituted by argenine residues and for this reason is not ubiquitinated anymore, is not affected; however, internalization still depends on an active ubiquitin system [354,355]. A specific region in the cytosolic tail of the GHR, termed the ubiquitin-dependent endocytosis (UbE) motif, is essential for both ubiquitination and internalization of the receptor [355]. It consists of the amino acid sequence DSWVEFIELD. Mutation of the phenylalanine residue into alanine prevents GHR (F327A) ubiquitination, incorporation into clathrin coated pits and subsequent internalization [355,356].

Beside the UbE motif, the GHR contains a di-leucine motif at position 347–348. This motif is inactive in the full length GHR, but can function in ubiquitin-independent internalization of the GHR (1–349) truncation mutant [357]. In contrast to the internalization, sorting to the lysosome still depends on an intact UbE motif in the GHR (1–349) mutant [358]. This finding indicates that the di-leucine motif is not sufficient for transport to the lysosome and GHR probably uses the UbE-motif at the endosome for sorting into MVB for lysosomal degradation. Possibly a ubiquititin-dependent sorting mechanism is involved in this step, similar to the ESCRT complex described in the paragraph ‘REGULATION OF MEMBRANE PROTEINS’ in this section (p. 38).

Also the 26S proteasome is involved in the GHR downregulation. GHR internalization requires proteasomal action in addition to an active ubiquitin system [354]. Specific proteasomal inhibitors block the internalization of the full length GHR, whereas this effect is lost when the GHR is truncated after amino acid 369 [354]. These findings hint to the partial degradation of the GHR or the degradation of an associated protein before internalization can occur [358]. Furthermore, it has been demonstrated

that proteasomal inhibitors prevent lysosomal degradation of GHR (1–349) [358] and cause the recycling of this truncated GHR [359].

Taken together, the ubiquitin-proteasome system is involved in two steps of the GHR downregulation. First, it is necessary for the internalization step at the cell surface and second, it is essential for sorting of the receptor to the lysosome.

# Growth Hormone Interacting Proteins

## Small Glutamine-Rich TPR-Containing Protein

Small glutamine-rich tetratricopeptide repeat (TPR) containing protein (SGT) shows some features similar to U-box E3 ligase CHIP. SGT, like CHIP, contains a TPR domain, through which it interacts with the C-terminus of Hsc70/Hsp70 [360]. In addition, SGT acts as a negative regulator of protein folding, similar to CHIP [361]. Unlike CHIP no U-box is present in SGT and also other functional domains that might link it to ubiquitination are not known to date.

SGT was initially identified as a protein interacting with envelope proteins of two viruses, namely human immunodeficiency virus type 1 and parvovirus H-1 [362,363]. Tobaben *et al.* have described SGT as part of a trimeric synaptic chaperone machine that also includes hsc70 and cysteine string protein (CSP) [364]. CSP, which contains a DNA-J domain, is thought to have an important function in the exocytic release of neurotransmitters, hormones, and enzyme precursors [365]. Overexpression of SGT in cultured neurons inhibits neurotransmitter release, suggesting that the CSP/Hsc70/SGT complex is important for maintaining normal synapses. Furthermore, SGT interacts with β-amyloid peptide (Aβ) [366], a primary component of the extracellular senile plaques characteristic of Alzheimer disease [367]. Double stranded RNA inhibition of SGT in *C. elegans* results in suppression of toxicity associated with Aβ expression [366].

### *TPR Motifs*

One important structural feature of SGT is its TPR domain, which has been found in a wide variety of proteins since it has been discovered in 1990 [368,369]. TPR-containing proteins are involved in a diverse spectrum of cellular functions with the majority of them participating in cell cycle control, transcription and splicing events, protein transport, regulatory phosphate turnover, and protein folding. Currently, more than 50 proteins are known to contain a TPR domain, which are present in organisms as

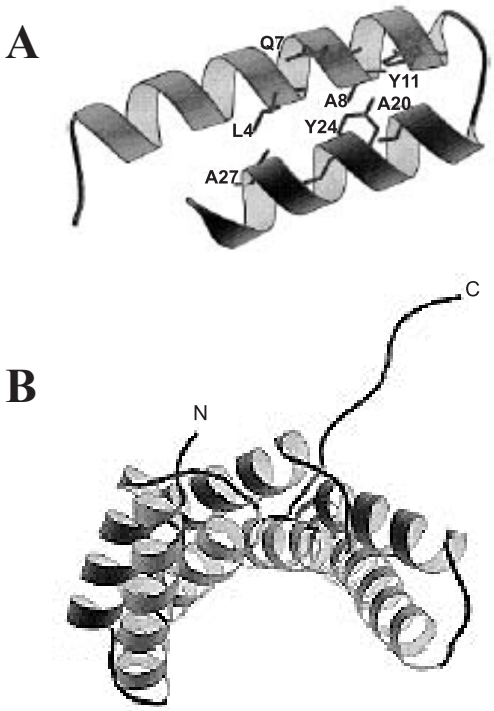



**Figure 8. Ribbon representation of the TPR domain of PP5.**
(**A**) Side view of the first TPR motif of PP5 shows the highly conserved TPR consensus residues in the inter-helix space. The residues are labeled by using the single-letter code and numbering refers to the TPR motif consensus numbering (see text).
(**B**) View parallel to the helical axis illustrates that the surface of the helical groove is formed by helix A. Helix A and helix B are represented by light gray and dark gray, respectively. This figure has been adapted from ref. [370].

diverse as bacteria and humans [370]. The number of TPR motifs within a TPR domain varies between different proteins, and there is no preferential positioning along the primary sequence of the protein. The TPR motif is defined as a degenerated, 34 amino acid repeat that is often arranged in a tandem repeat. Although degenerated, the motif demonstrates a conserved pattern of amino acid similarity and homology in terms of size, hydrophobicity, and spacing. In particular, eight amino acids show a comparatively high frequency of conservation generating a consensus at positions 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (A/S/L), and 32 (P/K/E) [369]. Sequence conservation outside these eight amino acids is only found between functionally equivalent TPR motifs. The secondary structure predictions of TPR motifs has been proposed to form two α-helical domains, A and B; domain A spans the conserved residues 4, 7, 8, 10, and 11, whereas domain B spans the consensus residues 20, 24, and 27 [371]. This prediction has been confirmed by the crystal structure for the PP5 N-terminal TPR domain [Figure 8 and ref. 372]. Each TPR motif is arranged in a parallel manner, such that the sequentially adjacent α-helices are anti-parallel. Within a

tandem array of TPR motifs, the packing of helices within and between adjacent TPR motifs is identical thus each α-helix shares two immediate α-helix neighbors. This regular repeat of α-helices generates a right-handed helical confirmation that creates an amphiphatic channel. Das *et al.* propose that tandem TPR motifs fold into a right-handed super-helix with a helical repeat of approximately seven TPR motifs, a pitch of 60 Å and a width of 42 Å. The inside of the TPR helix would be formed by helix A of each TPR motif, with the B helix located on the outside of the helix. Five to six TPR motifs could accommodate an α-helix of a target protein [372]. To date there is no evidence that TPR motifs of different TPR-containing proteins interact directly, but they are important for protein-protein interactions between a TPR-containing protein and one or more non-TPR proteins. Four major types of complexes that involve TPR proteins have been identified: the anaphase promoting complex, the transcription repression complex, the protein import complex of the peroxisomes and mitochondria, and the molecular chaperone complex.

The anaphase promoting complex, which includes the TPR proteins CDC16, CDC23, and CDC27, is a multi-subunit RING domain E3 involved in the degradation of cell cycle regulators [373-377]. A transcription repressor complex in *Saccharomyces cerevisiae* involving the TPR proteins SSN6/CYC8 and Tup1 recognizes specific DNA-binding proteins and mediates the repression of otherwise unrelated promotors [378]. The peroxisomal import receptor complex involving the TPR proteins PAS8/PAS10/PXR1 binds to proteins and transports them across peroxisomal membranes [379-382]. In mitochondria the same tasks are performed by the TPR proteins MAS70/MOM72 and MAS20/MOM19 [383,384].

The molecular heat shock proteins hsc70 and hsp90 interact with a number of co-chaperones that contain TPR motifs. For example, the co-chaperone STI1, a stress inducible protein first described in yeast, is part of a multi-protein chaperone complex, in which STI1 links Hsp70 and Hsp90. This Hsp70/STI1/Hsp90 complex is involved in the maturation of steroid receptor complexes. CHIP is another example of a TPR-containing protein that functions as a negative regulator of Hsc70 function. In addition, CHIP contains a U-box and shows E3 ligase function (see section 'UBIQUITIN-PROTEASOME SYSTEM' p. 31). Furthermore, sgt1, another TPR-containing protein, was identified because of its ability to interact with Skp1, a subunit of the SCF E3 ligase complex. Taken together, several of these co-chaperones have been linked with the ubiquitin-proteasome system. Further studies will be needed to characterize their function.

## Scythe

Already a decade ago, the Reaper interacting protein Scythe was identified. Scythe, also known as Bat3, Bag6 or Chap2 in yeast, was originally identified as one of the genes located within the human major histocompatibility complex III [385]. It encodes a 150 kDa proline-rich protein with short tracts of polyproline, polyglycine, and charged amino acids. The N-terminus bears a ubiquitin-like domain that shows 37% identity and 54% similarity to ubiquitin. The central region is cysteine/histidine-rich and is an imperfect copy of the canonical zinc finger motif. The functional significance of these domains is still unclear. A Bat3 gene has been isolated from rat and shown to be expressed in most adult tissue but predominantly in testis [386]. Green fluorescent protein-tagged rat Bat3 is mostly localized in the cytoplasm of COS cells [386]. In a different study, however, human scythe showed nuclear localization in HeLa cells, due to a nuclear localization signal that resides at the C-terminus of the protein [387]. Induction of apoptosis in these cells by staurosporine does not affect the nuclear localization of Scythe [387].

The first evidence that Scythe is involved in apoptosis regulation came in 1998 with the finding that *Xenopus* Scythe can interact with *Drosophila* Reaper. Immunodepletion of Scythe from *Xenopus* egg extracts completely prevented Reaper-induced apoptosis without affecting apoptosis triggered by activated caspases. However, Scythe lacking the N-terminal domain is able to induce apoptosis also in the absence of Reaper [215]. Tress *et al.* show evidence that Scythe sequesters a positive regulator of apoptosis that, when not bound by Scythe, can trigger cytochrome c release from purified mitochondria in the absence of other cytosolic components. This cytochrome c-releasing activity can be liberated by binding Reaper to Scythe [Figure 9 and ref. 388].

Recently, Scythe has been identified as a modulator of Hsc70/Hsp70, capable of

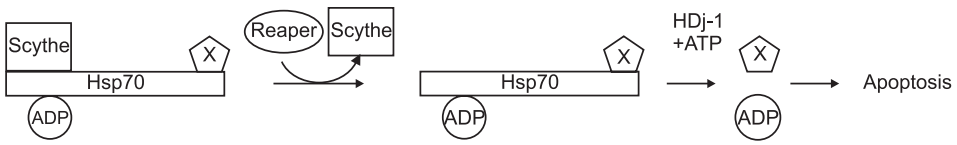



**Figure 9. Model for Reaper/Scythe function.**
Reaper serves as a ligand to trigger Scythe dissociation from the Hsp70 complex. According to this model, 'X' is released in its native form and can trigger mitochondrial cytochrome c release and caspase activation. This figure has been adopted from ref. [389].

inhibiting chaperone-mediated protein folding. Reaper on the other hand is able to reverse this inhibitory effect of Scythe [389]. These findings might suggest an important role of protein folding in the control of apoptosis.

BAG-1 is the first described negative regulator of Hsc70/Hsp70 function. Interestingly, BAG-1 has been first identified as an anti-apoptotic factor due to its capacity to interact with Bcl-2 and shows anti-apoptotic functions [390,391]. Furthermore, this protein also contains an N-terminal ubiquitin-like domain that is recognized by components of the proteasome [390]. In addition, it comprises a BAG-domain, a conserved C-terminal motif of approximately 50 amino acids that allows proteins to interact with and regulate Hsc70/Hsp70 chaperones [392]. Hsc70/Hsp70 possesses an N-terminal ATPase domain and a C-terminal substrate-binding domain. BAG family proteins bind to the ATPase domain with high affinity. Recently, it was shown that BAG-1 is involved in protein-quality control where it functions as a degradation factor [393,394].

One may speculate about a similar function for Scythe. So far, no data available indicate that the ubiquitin-like domain is recognized by the proteasome. On the other hand, Scythe possesses a C-terminal BAG domain through which it can interact with Hsc70. It is suggested that BAG-family proteins have either a positive or negative influence on substrate folding *in vivo*, depending on the ratios of other co-chaperones and the specific substrate protein [395]. Thus, Scythe might function both in apoptosis and protein quality control.

## TACE

The tumor necrosis factor-α converting enzyme, TACE, also referred to as ADAM17 or CD156q, is a member of the ‘a disintegrin and metalloprotease’ (ADAM) family. This family falls within the metzinicin superfamily, which also includes the matrix metalloproteases [396]. Members of the ADAM family are implicated in diverse


**Figure 10. Domain structure of TACE.**
TACE is synthesized as a precursor, which includes a prodomain, a catalytic metallopotease domain containing the Zn-binding site (indicated as a notch), a disintegrin domain (DIS), a cysteine-rich domain (Cys-rich), a transmembrane domain (TM), and a cytoplasmic tail (Cyto).

processes, including sperm-egg binding and fusion, myoblast fusion, protein ectodomain processing or shedding of cytokines, cytokine receptors, adhesion proteins, and other extracellular domains [397]. They are approximately 750 amino acid long, type I transmembrane proteins with a conserved domain structure, consisting of an N-terminal signal sequence followed by a prodomain, a metalloprotease domain (with or without a catalytic consensus), a disintegrin domain, a cysteine-rich domain usually containing an EGF repeat, and finally a transmembrane domain and a cytoplasmic tail [Figure 10 and ref. 398]. So far, 29 ADAM cDNAs have been identified in organisms ranging from protozoans to mammalians. Among these 29 ADAMs only 17 have the conserved catalytical consensus sequence, HEXGHXXGXXHD (single letter amino acid code; X represents any amino acid), which is the predicted catalytic site of an active zinc-dependent metalloprotease [396]. The other ADAMs possess one or more residues in this region that are incompatible with metalloprotease activity and therefore, they are thought to lack protease activity [398]. The X-ray structure of the catalytic domain of TACE shows that the zinc environment and the placement of the major structural elements closely resemble those of the snake venom member of the ADAM family [399]. In addition, a number of loops protrude outward from the core, which might be involved in the regulation or substrate interactions.

As the name already indicates, the disintegrin domain is beside the metalloprotease domain the second characteristic feature of members of the ADAM family. For several ADAMs it has been demonstrated that they can interact with integrins via their disintegrin domain [400]. However, it is not clear, whether all ADAMs are capable to interact with one or more integrins via their disintegrin domain or whether this domain might also be involved in other protein interactions. The role of the disintegrin domain in TACE is not known.

The prodomain of catalytically active ADAMs is considered to act as an inhibitor of the protease via a cysteine switch mechanism, in which a free cysteine residue from the prodomain coordinates the zinc ion in the active site of the protease [401]. The prodomain of several ADAM proteins, like TACE, have been shown to be constitutively cleaved by a furine-type proprotein convertase as the ADAM proteins progress through the secretory pathway [402]. Beside its role as an inhibitor of the protease domain, the prodomain appears to be important for the proper maturation and intracellular transport of at least some ADAMs [402-404]. The cysteine-rich domain functions in substrate recognition [405] and is necessary for shedding of some substrates but not for others. The transmembrane domain is responsible for membrane anchoring the ADAMs and aligning

them with their substrate in the membrane, because effective engagement of the substrate apparently requires membrane anchoring of both the substrate and the enzyme in some cases [406]. The cytosolic domain is not necessary for PMA-induced shedding. But there are reports indicating that in normal cells other stimulatory or inhibitory agents regulate the activity of TACE via its cytoslic tail. The cytoplasmic domain of many ADAMs contains SH3 domain binding sites; several of these interact with SH3 domain containing proteins including Src family members. It is still poorly understood how these signaling molecules and protein kinase C, which is activated upon phorbol ester stimulation, are involved in the regulation of TACE. Studies on the localization of TACE indicate that the major pool of TACE resides in an intracellular perinuclear compartment, with some diffuse localization consistent with either surface or ER staining [407]. Only processed TACE appears at the cell surface [408].

The characteristics that render a protein a substrate for TACE are still not clear. The sequences cleaved in various substrates are highly variable, even though there is a preference for valine as the residue contributing the amide of the cleaved peptide bond. The crystal structure of the substrate binding cleft of TACE does not suggest a strong interaction between the enzyme and its substrate TNF [399]. Therefore, interactions more distal to the cleavage site are probably required, and at least in one instance the cysteine-rich domain of TACE appears to be important for substrate recognition.

## Outline of the Thesis

The effectiveness of GH depends not only on its concentration in the circulation, but also on the availability of GHR molecules at the cell surface of target cells. The turnover of GHR molecules at the plasma membrane is rapid with a half-life of approximately 75 min and is mainly determined by ubiquitin system-dependent internalization. This process depends on an intact ubiquitin system and the ubiquitin-dependent endocytosis (UbE) motif in the cytosolic tail of the GHR. The UbE motif might serve as the docking site of the ubiquitin system, the endocytotic machinery, or both. Proteolysis of the extracellular domain results in the release of the GHBP and determines the GHR turnover to a lesser extent. In this thesis, we focused on the identification and characterization of proteins involved in GHR turnover at the cell surface.

We identified several proteins that are capable to interact with the UbE motif of the GHR by using the part of the cytosolic tail containing the UbE motif in a GST-pulldown

assay. By this means, we identified SGT, a TPR domain containing protein, which interacted through the first TPR motif with the UbE motif of the GHR. This interaction was independent of the ubiquitin system, indicating that the ubiquitin system may act downstream of the GHR-SGT interaction (Chapter II). A second very interesting protein was isolated by GST-pulldown experiments, called Scythe. This protein contains a ubiquitin-like domain at its N-terminus and binds through its C-terminal BAG domain to Hsc70. Originally identified as an anti-apoptotic protein, we localized the protein to mitochondria, the nucleus, and peri-nuclear area. Induction of apoptosis by UVC treatment, left the nuclear and peri-nulcear localization unchanged, whereas the mitochondria were destroyed by the UV treatment. This localization was independent of proteasomal inhibitors. However, treatment with cyclohexamide resulted in drastic reduction of hScythe localization to mitochondria, while the nuclear and perinuclear staining remained unchanged (Chapter III). The third protein we identified was large proline rich protein 130 kDa (LRP130). No further research was carried out. In Chapter IV we show that GHR interacted directly with its sheddase TACE, which is responsible for the proteolytic release of the GHBP. We showed that interaction still occurred in the presence of GH, a condition under which shedding by TACE is inhibited (Chapter IV).

# References


1. Evans, H. M., Long J.A. (1921) *Anat. Rec.* **21**, 61-63
2. Smith, P. E. (1930) *Am. J. Anat.* **45**, 205-273
3. Li, C. H., Evans, H. M., and Simpson, M. E. (1945) *J. Biol. Chem.* **159**, 353-366
4. Bennett, L. L., Weinberger, H., Wscamilla, R., *et al.* (1950) *J. Clin. Endocrinol.* **10**, 492-495
5. Li, C. H., and Papkoff, H. (1956) *Science* **124**, 1293-1294
6. Li, C. H. (1957) *Fed. Proc.* **16**, 775-783
7. Salmon, W. D., and Daughaday, W. H. (1957) *J. Lab. Clin. Med.* **49**, 825-826
8. D'Ercole, A. J., Applewhite, G. T., and Underwood, L. E. (1980) *Dev. Biol.* **75**, 315-328
9. Isaksson, O. G., Jansson, J. O., and Gause, I. A. (1982) *Science* **216**, 1237-1239
10. Green, H., Morikawa, M., and Nixon, T. (1985) *Differentiation* **29**, 195-198
11. Le Roith, D., Bondy, C., Yakar, S., *et al.* (2001) *Endocrinol Rev.* **22**, 53-74
12. Casanueva, F. F. (1992) *Endcrinol. Metab. Clin. North Am.* **21**, 483-517
13. Davidson, M. B. (1987) *Endocrinol. Rev.* **8**, 115-131
14. Touw, I. P., De Koning, J. P., Ward, A. C., *et al.* (2000) *Mol. Cell Endocrinol.* **160**, 1-9
15. Okada, S., and Kopchick, J. J. (2001) *Trends Mol. Med*. **7**, 126-132
16. Miller, L., and Eberhardt, N. (1983) *Endocrinol. Rev.* **4**, 97-129
17. Goffin, V., Shiverick, K. T., Kelly, P. A., *et al.* (1996) *Endocrinol. Rev.* **17**, 385-410
18. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255**, 306-312
19. Waters, M. J., Shang, C. A., Behncken, S. N., *et al.* (1999) *Clin. Exp. Pharmacol. Physiol.* **26**, 760-764

20. Frohman, L. A., Downs, T. R., and Chomczynski, P. (1992) *Front Neuroendocrinol.* **13**, 344-405
21. Miller, J. D., Tannenbaum, G. S., Colle, E.*, et al.* (1982) *J. Clin. Endocrinol. Metab.* **55**, 989-994
22. Jansson, J. O., Albertsson Wikland, K., Eden, S.*, et al.* (1982) *Acta Physiol. Scand.* **114**, 261-265
23. Clemmons, D. R., and Van Wyk, J. J. (1984) *Clin. Endocrinol. Metab.* **13**, 113-143
24. Shimasaki, S., Shimonaka, M., Zhang, H. P.*, et al.* (1991) *J. Biol. Chem.* **266**, 10646-10653
25. Jones, J. I., and Clemmons, D. R. (1995) *Endocrinol. Rev.* **16**, 3-34
26. Muller, E. E., Locatelli, V., and Cocchi, D. (1999) *Physiol. Rev.* **79**, 511-607
27. Wallenius, K., Sjogren, K., Peng, X. D.*, et al.* (2001) *Endcrinol.* **142**, 4762-4770
28. Beck, T., Schmidt, A., and Hall, M. N. (1999) *J. Cell Biol.* **146**, 1227-1237
29. Bowers, C. Y., Momany, F. A., Reynolds, G. A.*, et al.* (1984) *Endocrinol.* **114**, 1537-1545
30. Bowers, C. Y., Momany, F., Reynolds, G. A.*, et al.* (1980) *Endocrinol.* **106**, 663-667
31. Howard, A. D., Feighner, S. D., Cully, D. F.*, et al.* (1996) *Science* **273**, 974-977
32. Kojima, M., Hosoda, H., Date, Y.*, et al.* (1999) *Nature* **402**, 656-660
33. Casanueva, F. (1992) *Endocrinol. Metab. Clin. North Amer.* **21**, 483-517
34. Isaksson, O. G., Eden, S., and Jansson, J. O. (1985) *Annu. Rev. Physiol.* **47**, 483-499
35. Rudman, D., Feller, A. G., Nagraj, H. S.*, et al.* (1990) *N. Engl. J. Med.* **323**, 1-6
36. Rosenfeld, R. G., Wilson, D. M., Dollar, L. A.*, et al.* (1982) *J. Clin. Endocrinol. Metab.* **54**, 1033-1038
37. Sonksen, P. H., Salomon, F., and Cuneo, R. (1991) *Horm Res* **36**, 27-31
38. Carroll, P. V., and Christ, E. R. (1998) *J. Clin. Endocrinol. Metab.* **83**, 382-395
39. Kostyo, J. L. (1968) *Ann. NY Acad. Sci.* **148**, 389-407
40. Jiang, Z. M., He, G. Z., Zhang, S. Y.*, et al.* (1989) *Ann. Surg.* **210**, 513-524
41. Gilpin, D. A., Barrow, R. E., Rutan, R. L.*, et al.* (1994) *Ann. Surg.* **220**, 19-24
42. Krentz, A. J., Koster, F. T., Crist, D. M.*, et al.* (1993) *J. Acquired Immune Defic. Syndr.* **6**, 245-251
43. Clark, R. (1997) *Endocr Rev* **18**, 157-179
44. Welniak, L. A., Sun, R., and Murphy, W. J. (2002) *J. Leukocyte Biol.* **71**, 381-387
45. Yoshizato, H., Fujikawa, T., Soya, H.*, et al.* (1998) *Endcrinol.* **139**, 2545-2551
46. Cheek, D. B., and Hill, D. E. (1974) (Knobil, E., and Sawyer, W. H., eds.) *American Physiology Society* **4(2)**, 159-185
47. Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6934-6938
48. Carter-Su, C., Schwartz, J., and Smit, L. S. (1996) *Annu. Rev. Physiol.* **58**, 187-207
49. Argetsinger, L. S., and Carter-Su, C. (1996) *Physiol. Rev.* **76**, 1089-1107
50. Kelly, P. A., Djiane, J., Postel-Vinay, M. C.*, et al.* (1991) *Endocrinol. Rev* **12**, 235-251
51. Baumgartner, J. W., Wells, C. A., Chen, C. M.*, et al.* (1994) *J. Biol. Chem.* **269**, 29094-29101
52. Ihle, J. N., Witthuhn, B. A., Quelle, F. W.*, et al.* (1995) *Annu. Rev. Immunol* **13**, 369-398
53. Argetsinger, L. S., Campbell, G. S., Yang, X. N.*, et al.* (1993) *Cell* **74**, 237-244
54. Smit, L. S., Meyer, D. J., Billestrup, N.*, et al.* (1996) *Mol Endocrinol* **10**, 519-533
55. Johnston, J. A., Kawamura, M., Kirken, R. A.*, et al.* (1994) *Nature* **370**, 151-153
56. Hellgren, G., Jansson, J. O., Carlsson, L. M. S.*, et al.* (1999) *Growth Horm. IGF Res.* **9**, 212-218
57. Carter-Su, C., King, A. P., Argetsinger, L. S.*, et al.* (1996) *Endocrinol. J.* **43**, S65-70
58. Hellgren, G., Albertsson-Wikland, K., Billig, H.*, et al.* (2001) *J. Interferon Cytokine Res.* **21**, 75-83
59. Frank, S. J., Gilliland, G., Kraft, A. S.*, et al.* (1994) *Endocrinol.* **135**, 2228-2239
60. Sotiropoulos, A., Perrot Applanat, M., Dinerstein, H.*, et al.* (1994) *Endocrinol.* **135**, 1292-1298
61. Murakami, M., Narazaki, M., Hibi, M.*, et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11349-11353
62. DaSilva, L., Howard, O. M., Rui, H.*, et al.* (1994) *J. Biol. Chem.* **269**, 18267-18270

63. Tanner, J. W., Chen, W., Young, R. L., *et al.* (1995) *J. Biol. Chem.* **270**, 6523-6530
64. Dinerstein, H., Lago, F., Goujon, L., *et al.* (1995) *Mol. Endocrinol.* **9**, 1701-1707
65. Leung, D. W., Spencer, S. A., Cachianes, G., *et al.* (1987) *Nature* **330**, 537-543
66. Hughes, J. P., Simpson, J. S., and Friesen, H. G. (1983) *Endocrinol.* **112**, 1980-1985
67. Hocquette, J. F., Postel-Vinay, M. C., Djiane, J., *et al.* (1990) *Endocrinol.* **127**, 1665-1672
68. Harding, P. A., Wang, X. Z., Kelder, B., *et al.* (1994) *Mol. Cell Endocrinol* **106**, 171-180
69. DeVos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255**, 306-312
70. Szecowka, J., Tai, L. R., and Goodman, H. M. (1990) *Endocrinol.* **126**, 1834-1841
71. Godowski, P. J., Leung, D. W., Meacham, L. R., *et al.* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8083-8087
72. Ross, R. J., Esposito, N., Shen, X. Y., *et al.* (1997) *Mol Endocrinol* **11**, 265-273
73. Amit, T., Bergman, T., Dastot, F., *et al.* (1997) *J. Clin. Endocrinol. Metab.* **82**, 3813-3817
74. Urbanek, M., Macleod, J. N., Cooke, N. E., *et al.* (1992) *Mol Endocrinol* **6**, 279-287
75. Sobrier, M. L., Duquesnoy, P., Duriez, B., *et al.* (1993) *FEBS Lett* **319**, 16-20
76. Urbanek, M., Russell, J. E., Cooke, N. E., *et al.* (1993) *J. Biol. Chem.* **268**, 19025-19032.
77. Ayling, R. M., Ross, R., Towner, P., *et al.* (1997) *Nature Genet.* **16**, 13-14
78. Rosenbloom, A. L., and Guevara-Aguirre, J. (1998) *Trends Endocrinol. Metab.* **9**, 276-283
79. Laron, Z., Pertzelan, A., and Mannheimer, S. (1966) *Isr. J. Med. Sci.* **2**, 152-155
80. Rose-John, S., and Heinrich, P. C. (1994) *Biochem. J.* **300 ( Pt 2)**, 281-290
81. Baumann, G., Amburn, K., and Shaw, M. (1988) *Endocrinol.* **122**, 976-984
82. Baumann, G., Amburn, K. D., and Buchanan, T. A. (1987) *J. Clin. Endocrinol. Metab.* **64**, 657-660
83. Cunningham, B. C., Ultsch, M., deVos, A. M., *et al.* (1991) *Science* **254**, 821-825
84. Baumann, G. (2001) *J. Pediatr. Endocrinol. Metab.* **14**, 355-375
85. Baumbach, W. R., Horner, D. L., and Logan, J. S. (1989) *Genes Dev.* **3**, 1199-1205
86. Edens, A., Southard, J. N., and Talamantes, F. (1994) *Endocrinol.* **135**, 2802-2805
87. Zhou, Y., He, L., and Kopchick, J. J. (1994) *Receptor* **4**, 223-227
88. Sotiropoulos, A., Guojon, L., Simonin, G., *et al.* (1993) *Endocrinol.* **132**, 1863-1865
89. Saito, Y., Ikebuchi, H., Yamazaki, T., *et al.* (1995) *J. Biochem.* **118**, 521-525
90. Guan, R., Zhang, Y., Jiang, J., *et al.* (2001) *Endocrinol.* **142**, 1137-1147
91. Trivedi, B., and Daughaday, W. H. (1988) *Endocrinol.* **123**, 2201-2206
92. Saito, Y., Takagi, K., Teshima, R., *et al.* (1998) *Mol. Cell Endocrinol.* **146**, 197-205
93. Amit, T., Bar-Am, O., Dastot, F., *et al.* (1999) *Endocrinol.* **140**, 266-272
94. Zhang, Y., Guan, R., Jiang, J., *et al.* (2001) *J. Biol. Chem* **276**, 24565-24573
95. Zhang, Y., Jiang, J., Black, R. A., *et al.* (2000) *Endocrinol.* **141**, 4342-4348
96. Conte, F., Salles, J. P., Raynal, P., *et al.* (2002) *Biochem. Biophys. Res. Commun.* **290**, 851-857
97. Baumann, G., and Frank, S. J. (2002) *J. Endocrinol.* **174**, 361-368
98. Cunningham, B. C., Jhurani, P., Ng, P., *et al.* (1989) *Science* **243**, 1330-1336
99. Cunningham, B. C., and Wells, J. A. (1989) *Science* **244**, 1081-1085
100. Waters, M. J., Rowlinson, S. W., Clarkson, R. W., *et al.* (1994) *Proc. Soc. Exp. Biol. Med.* **206**, 216-220
101. Fuh, G., Cunningham, B. C., Fukunaga, R., *et al.* (1992) *Science* **256**, 1677-1680
102. Chen, C. M., Brinkworth, R., and Waters, M. J. (1997) *J. Biol. Chem.* **272**, 5133-5140
103. Mellado, M., Rodriguezfrade, J. M., Kremer, L., *et al.* (1997) *J. Biol. Chem.* **272**, 9189-9196
104. Harding, P. A., Wang, X., Okada, S., *et al.* (1996) *J. Biol. Chem.* **271**, 6708-6712
105. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinol.* **143**, 1243-1252
106. Gent, J., van Kerkhof, P., Roza, M., *et al.* (2002) *Proc. Nat. Acad. Sci. USA*

107. Silva, C. M., Day, R. N., Weber, M. J., *et al.* (1993) *Endocrinol.* **133**, 2307-2312
108. Campbell, G. S., Christian, L. J., and Carter Su, C. (1993) *J. Biol. Chem.* **268**, 7427-7434
109. Moller, C., Hansson, A., Enberg, B., *et al.* (1992) *J.Biol.Chem.* **267**, 23403-23408
110. Silva, C. M., Weber, M. J., and Thorner, M. O. (1993) *Endocrinol.* **132**, 101-108
111. Winston, L. A., and Bertics, P. J. (1992) *J.Biol.Chem.* **267**, 4747-4751
112. Carter Su, C., Stubbart, J. R., Wang, X. Y., *et al.* (1989) *J.Biol.Chem.* **264**, 18654-18661
113. VanderKuur, J. A., Wang, X. Y., Zhang, L. Y., *et al.* (1994) *J. Biol. Chem.* **269**, 21709-21717
114. Pawson, T., and Schlessinger, J. (1993) *Curr. Biol.* **3**, 434-442
115. Darnell, J., Kerr, I., and Stark, G. (1994) *Science* **264**, 1415-1421
116. Herrington, J., Smit, L. S., Schwartz, J., *et al.* (2000) *Oncogene* **19**, 2585-2597
117. Vanderkuur, J. A., Butch, E. R., Waters, S. B., *et al.* (1997) *Endocrinol.* **138**, 4301-4307
118. Cobb, M., and Goldsmith, E. (1995) *J. Biol. Chem.* **270**, 14843-14846
119. Argetsinger, L. S., Hsu, G. W., Myers, M. G., Jr., *et al.* (1995) *J. Biol. Chem.* **270**, 14685-14692
120. Souza, S. C., Frick, G. P., Yip, R., *et al.* (1994) *J. Biol. Chem.* **269**, 30085-30088
121. Yamauchi, T., Kaburagi, Y., Ueki, K., *et al.* (1998) *J. Biol. Chem.* **273**, 15719-15726
122. Argetsinger, L. S., Norstedt, G., Billestrup, N., *et al.* (1996) *J.Biol.Chem.* **271**, 29415-29421
123. Cheatham, B., Vlahos, C. J., Cheatham, L., *et al.* (1994) *Mol. Cell Biol.* **14**, 4902-4911
124. Moutoussamy, S., Kelly, P. A., and Finidori, J. (1998) *Eur. J. Biochem.* **255**, 1-11
125. Slootweg, M. C., De Groot, R. P., Herrmann-Erlee, M. P., *et al.* (1991) *J. Mol. Endocrinol.* **6**, 179-188
126. Gaur, S., Yamaguchi, H., and Goodman, H. M. (1996) *Am. J. Physiol.* **270**, C1485-C1492
127. Gaur, S., Yamaguchi, H., and Goodman, H. M. (1996) *Am. J. Physiol.* **270**, C1478-C1484
128. Billestrup, N., Bouchelouche, P., Allevato, G., *et al.* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2725-2729
129. Hackett, R. H., Wang, Y. D., Sweitzer, S., *et al.* (1997) *J. Biol. Chem.* **272**, 11128-11132
130. Kim, S. O., Jiang, J., Yi, W. S., *et al.* (1998) *J. Biol. Chem.* **273**, 2344-2354
131. Alves dos Santos, C. M., van Kerkhof, P., and Strous, G. J. (2001) *J. Biol. Chem.* **276**, 10839-10846
132. Stofega, M. R., Herrington, J., Billestrup, N., *et al.* (2000) *Mol Endocrinol* **14**, 1338-1350
133. Stofega, M. R., Wang, H., Ullrich, A., *et al.* (1998) *J. Biol. Chem.* **273**, 7112-7117
134. Ohnishi, H., Kubota, M., Ohtake, A., *et al.* (1996) *J. Biol. Chem.* **271**, 25569-25574
135. Stofega, M. R., Argetsinger, L. S., Wang, H., *et al.* (2000) *J. Biol. Chem.* **275**, 28222-28229
136. Starr, R., Willson, T. A., Viney, E. M., *et al.* (1997) *Nature* **387**, 917-921
137. Krebs, D. L., and Hilton, D. J. (2000) *J Cell Sci* **113**, 2813-2819
138. Matsumoto, A., Masuhara, M., Mitsui, K., *et al.* (1997) *Blood* **89**, 3148-3154
139. Naka, T., Narazaki, M., Hirata, M., *et al.* (1997) *Nature* **387**, 924-929
140. Endo, T. A., Masuhara, M., Yokouchi, M., *et al.* (1997) *Nature* **387**, 921-924
141. Zhang, J. G., Farley, A., Nicholson, S. E., *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2071-2076
142. Ungureanu, D., Saharinen, P., Junttila, I., *et al.* (2002) *Mol. Cell Biol.* **22**, 3316-3326
143. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) *Br. J. Cancer* **26**, 239-257
144. Vaux, D. L., Haecker, G., and Strasser, A. (1994) *Cell* **76**, 777-779
145. Raff, M. C. (1992) *Nature* **356**, 397-400
146. Thompson, C. B. (1995) *Science* **267**, 1456-1462
147. Arends, M. J., and Wyllie, A. H. (1991) *Int. Rev. Exp. Pathol.* **32**, 223-254
148. Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100**, 57-70
149. Jellinger, K. A. (2001) *J. Cell. Mol. Med.* **5**, 1-17
150. Hacker, G. (2000) *Cell Tissue Res.* **301**, 5-17

151. Nicholson, D. W. (1999) *Cell Death Differ.* **6**, 1028-1042
152. Budihardjo, I., Oliver, H., Lutter, M., *et al.* (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 269-290
153. Stennicke, H. R., and Salvesen, G. S. (1998) *Biochim. Biophys. Acta* **1387**, 17-31
154. Kischkel, F. C., Hellbardt, S., Behrmann, I., *et al.* (1995) *Embo J.* **14**, 5579-5588
155. Medema, J. P., Scaffidi, C., Kischkel, F. C., *et al.* (1997) *Embo J.* **16**, 2794-2804
156. Salvesen, G. S., and Dixit, V. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 10964-10967
157. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., *et al.* (1995) *Cell* **81**, 505-512
158. Nomura, M., Shimizu, S., Ito, T., *et al.* (1999) *Cancer Res.* **59**, 5542-5548
159. Eskes, R., Desagher, S., Antonsson, B., *et al.* (2000) *Mol. Cell Biol.* **20**, 929-935
160. Suzuki, M., Youle, R. J., and Tjandra, N. (2000) *Cell* **103**, 645-654
161. Wei, M. C., Lindsten, T., Mootha, V. K., *et al.* (2000) *Genes Dev.* **14**, 2060-2071
162. Antonsson, B., Montessuit, S., Sanchez, B., *et al.* (2001) *J. Biol. Chem.* **276**, 11615-11623
163. Desagher, S., and Martinou, J. C. (2000) *Trends Cell Biol.* **10**, 369-377
164. Li, L. Y., Luo, X., and Wang, X. (2001) *Nature* **412**, 95-99
165. Acehan, D., Jiang, X., Morgan, D. G., *et al.* (2002) *Mol. Cell* **9**, 423-432
166. Rodriguez, J., and Lazebnik, Y. (1999) *Genes Dev.* **13**, 3179-3184
167. Cain, K., Bratton, S. B., Langlais, C., *et al.* (2000) *J. Biol. Chem.* **275**, 6067-6070
168. Cain, K., Brown, D. G., Langlais, C., *et al.* (1999) *J. Biol. Chem.* **274**, 22686-22692
169. Chittenden, T., Harrington, E. A., O'Connor, R., *et al.* (1995) *Nature* **374**, 733-736
170. Kelekar, A., and Thompson, C. B. (1998) *Trends Cell Biol.* **8**, 324-330
171. Adams, J. M., and Cory, S. (1998) *Science* **281**, 1322-1326
172. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) *Genes Dev.* **13**, 1899-1911
173. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 4997-5002
174. Cheng, E. H., Wei, M. C., Weiler, S., *et al.* (2001) *Mol. Cell* **8**, 705-711
175. Crook, N. E., Clem, R. J., and Miller, L. K. (1993) *J. Virol.* **67**, 2168-2174
176. Birnbaum, M. J., Clem, R. J., and Miller, L. K. (1994) *J. Virol.* **68**, 2521-2528
177. Duckett, C. S., Nava, V. E., Gedrich, R. W., *et al.* (1996) *Embo J.* **15**, 2685-2694
178. Roy, N., Mahadevan, M. S., McLean, M., *et al.* (1995) *Cell* **80**, 167-178
179. Hay, B. A., Wassarman, D. A., and Rubin, G. M. (1995) *Cell* **83**, 1253-1262
180. Uren, A. G., Beilharz, T., O'Connell, M. J., *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**, 10170-10175
181. Miller, L. K. (1999) *Trends Cell Biol.* **9**, 323-328
182. Hinds, M. G., Norton, R. S., Vaux, D. L., *et al.* (1999) *Nature Struct. Biol.* **6**, 648-651
183. Duckett, C. S., Li, F., Wang, Y., *et al.* (1998) *Mol. Cell Biol.* **18**, 608-615
184. Deveraux, Q. L., Roy, N., Stennicke, H. R., *et al.* (1998) *Embo J.* **17**, 2215-2223
185. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., *et al.* (1997) *Nature* **388**, 300-304
186. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol* **16**, 225-260
187. Chen, C., Edelstein, L. C., and Gelinas, C. (2000) *Mol. Cell Biol.* **20**, 2687-2695
188. Chu, Z. L., McKinsey, T. A., Liu, L., *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 10057-10062
189. Zong, W. X., Edelstein, L. C., Chen, C., *et al.* (1999) *Genes Dev.* **13**, 382-387
190. Jiang, Y., Woronicz, J. D., Liu, W., *et al.* (1999) *Science* **283**, 543-546
191. Joazeiro, C., and Weissman, A. M. (2000) *Cell* **102**, 549-552
192. Yang, Y., Fang, S., Jensen, J. P., *et al.* (2000) *Science* **288**, 874-877
193. Huang, H., Joazeiro, C. A., Bonfoco, E., *et al.* (2000) *J. Biol. Chem.* **275**, 26661-26664
194. Suzuki, Y., Nakabayashi, Y., and Takahashi, R. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 8662-8667
195. Verhagen, A. M., Ekert, P. G., Pakusch, M., *et al.* (2000) *Cell* **102**, 43-53

196. Du, C., Fang, M., Li, Y., *et al.* (2000) *Cell* **102**, 33-42
197. Chai, J., Du, C., Wu, J. W., *et al.* (2000) *Nature* **406**, 855-862
198. Srinivasula, S. M., Datta, P., Fan, X. J., *et al.* (2000) *J. Biol. Chem.* **275**, 36152-36157
199. Ekert, P. G., Silke, J., Hawkins, C. J., *et al.* (2001) *J. Cell Biol.* **152**, 483-490
200. Wu, G., Chai, J., Suber, T. L., *et al.* (2000) *Nature* **408**, 1008-1012
201. Liu, Z., Sun, C., Olejniczak, E. T., *et al.* (2000) *Nature* **408**, 1004-1008
202. Wilson, R., Goyal, L., Ditzel, M., *et al.* (2002) *Nature Cell Biol.* **4**, 445-450
203. Goyal, L., McCall, K., Agapite, J., *et al.* (2000) *Embo J.* **19**, 589-597
204. Holley, C. L., Olson, M. R., Colon-Ramos, D. A., *et al.* (2002) *Nature Cell Biol.* **4**, 439-444
205. Ryoo, H. D., Bergmann, A., Gonen, H., *et al.* (2002) *Nature Cell Biol.* **4**, 432-438
206. Hays, R., Wickline, L., and Cagan, R. (2002) *Nature Cell Biol.* **4**, 425-431
207. Yoo, S. J., Huh, J. R., Muro, I., *et al.* (2002) *Nature Cell Biol.* **4**, 416-424
208. Schwartz, L. M., Myer, A., Kosz, L., *et al.* (1990) *Neuron* **5**, 411-419
209. Grimm, L. M., Goldberg, A. L., Poirier, G. G., *et al.* (1996) *Embo J.* **15**, 3835-3844
210. Sadoul, R., Fernandez, P. A., Quiquerez, A. L., *et al.* (1996) *Embo J.* **15**, 3845-3852
211. White, K., Grether, M. E., Abrams, J. M., *et al.* (1994) *Science* **264**, 677-683
212. Nordstrom, W., Chen, P., Steller, H., *et al.* (1996) *Dev. Biol.* **180**, 213-226
213. Vucic, D., Kaiser, W. J., and Miller, L. K. (1998) *Mol. Cell Biol.* **18**, 3300-3309
214. Wing, J. P., Schreader, B. A., Yokokura, T., *et al.* (2002) *Nature Cell Biol.* **4**, 451-456
215. Thress, K., Henzel, W., Shillinglaw, W., *et al.* (1998) *Embo J.* **17**, 6135-6143
216. Evans-Storms, R. B., and Cidlowski, J. A. (1995) *J. Steroid Biochem. Mol. Biol.* **53**, 1-8
217. Kiess, W., and Gallaher, B. (1998) *Eur. J. Endocrinol.* **138**, 482-491
218. Haeffner, A., Deas, O., Mollereau, B., *et al.* (1999) *Eur. J. Immunol.* **29**, 334-344
219. Kolle, S., Stojkovic, M., Boie, G., *et al.* (2002) *Mol. Reprod. Dev.* **61**, 180-186
220. Jeay, S., Sonenshein, G. E., Kelly, P. A., *et al.* (2001) *Endocrinol.* **142**, 147-156.
221. Jeay, S., Sonenshein, G. E., Postelvinay, M. C., *et al.* (2000) *Mol Endocrinol* **14**, 650-661
222. Baixeras, E., Jeay, S., Kelly, P. A., *et al.* (2001) *Endocrinol.* **142**, 2968-2977
223. Tamatani, M., Che, Y. H., Matsuzaki, H., *et al.* (1999) *J. Biol. Chem.* **274**, 8531-8538
224. Wang, C. Y., Mayo, M. W., Korneluk, R. G., *et al.* (1998) *Science* **281**, 1680-1683
225. Lee, H., Arsura, M., Wu, M., *et al.* (1995) *J. Exp. Med.* **181**, 1169-1177
226. Guttridge, D. C., Albanese, C., Reuther, J. Y., *et al.* (1999) *Mol. Cell Biol.* **19**, 5785-5799
227. Costoya, J. A., Finidori, J., Moutoussamy, S., *et al.* (1999) *Endocrinol.* **140**, 5937-5943
228. Dinerstein, H., Lago, F., Goujon, L., *et al.* (1995) *Mol. Endocrinol.* **9**, 1701-1707
229. Kennedy, S. G., Wagner, A. J., Conzen, S. D., *et al.* (1997) *Genes Dev.* **11**, 701-713
230. Pianetti, S., Arsura, M., Romieu-Mourez, R., *et al.* (2001) *Oncogene* **20**, 1287-1299
231. Sherr, C. J. (1996) *Science* **274**, 1672-1677
232. Domenech-Santasusana, M., Carles, J., Goday, A., *et al.* (1994) *Ann. Oncol.* **5**, 659
233. Beuschlein, F., Strasburger, C. J., Siegerstetter, V., *et al.* (2000) *N. Engl. J. Med.* **342**, 1871-1876
234. Beyan, C., Ural, A. U., Cetin, T., *et al.* (1996) *Endocrinol. J.* **43**, 565-568
235. Bargou, R. C., Emmerich, F., Krappmann, D., *et al.* (1997) *J Clin. Invest.* **100**, 2961-2969
236. Bellavia, D., Campese, A. F., Alesse, E., *et al.* (2000) *Embo J.* **19**, 3337-3348
237. Guzman, M. L., Neering, S. J., Upchurch, D., *et al.* (2001) *Blood* **98**, 2301-2307
238. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67**, 425-479
239. Ciechanover, A., and Schwartz, A. L. (2002) *Hepatology* **35**, 3-6
240. Breitschopf, K., Bengal, E., Ziv, T., *et al.* (1998) *Embo J.* **17**, 5964-5973
241. Aviel, S., Winberg, G., Massucci, M., *et al.* (2000) *J. Biol. Chem.* **275**, 23491-23499
242. Reinstein, E., Scheffner, M., Oren, M., *et al.* (2000) *Oncogene* **19**, 5944-5950

243. Haas, A. L., and Siepma, T. J. (1997) *Faseb J.* **11**, 1257-1268
244. Pickart, C. M. (2000) *Trends Biochem. Sci.* **25**, 544-548
245. Koegl, M., Hoppe, T., Schlenker, S.*, et al.* (1999) *Cell* **96**, 635-644
246. Wilkinson, K. D. (2000) *Semin. Cell Dev. Biol.* **11**, 141-148
247. Rotin, D., Staub, O., and Haguenauer-Tsapis, R. (2000) *J. Membr. Biol.* **176**, 1-17
248. Spence, J., Sadis, S., Haas, A. L.*, et al.* (1995) *Mol. Cell Biol.* **15**, 1265-1273
249. Deng, L., Wang, C., Spencer, E.*, et al.* (2000) *Cell* **103**, 351-361
250. Cook, J. C., and Chock, P. B. (1992) *J. Biol. Chem.* **267**, 24315-24321
251. Finley, D., Ciechanover, A., and Varshavsky, A. (1984) *Cell* **37**, 43-55
252. Haas, A. L., and Siepmann, T. J. (1997) *Faseb J.* **11**, 1257-1268
253. Jentsch, S., Seufert, W., Sommer, T.*, et al.* (1990) *Trends Biochem. Sci.* **15**, 195-198
254. Mathias, N., Steussy, C. N., and Goebl, M. G. (1998) *J. Biol. Chem.* **273**, 4040-4045
255. Sommer, T., and Jentsch, S. (1993) *Nature* **365**, 176-179
256. Scheffner, M., Huibregtse, J. M., Vierstra, R. D.*, et al.* (1993) *Cell* **75**, 495-505
257. Huibregtse, J., Scheffner, M., Beaudenon, S.*, et al.* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2563-2567
258. Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995) *Nature* **373**, 81-83
259. Huibregtse, J. M., Scheffner, M., Beaudenon, S.*, et al.* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2563-2567
260. Huang, L., Kinnucan, E., Wang, G. L.*, et al.* (1999) *Science* **286**, 1321-1326
261. Nuber, U., and Scheffner, M. (1999) *J. Biol. Chem.* **274**, 7576-7582
262. Kumar, S., Kao, W. H., and Howley, P. M. (1997) *J. Biol. Chem.* **272**, 13548-13554.
263. Lu, P. J., Zhou, X. Z., Shen, M. H.*, et al.* (1999) *Science* **283**, 1325-1328
264. Plant, P. J., Lafont, F., Lecat, S.*, et al.* (2000) *J. Cell Biol.* **149**, 1473-1483
265. Staub, O., Gautschi, I., Ishikawa, T.*, et al.* (1997) *Embo J.* **16**, 6325-6336
266. Schild, L., Lu, Y., Gautschi, I.*, et al.* (1996) *Embo J.* **15**, 2381-2387
267. Freemont, P. S. (2000) *Curr. Biol.* **10**, R84-R87
268. Borden, K. L. B. (2000) *J. Mol. Biol.* **295**, 1103-1112
269. Zheng, N., Wang, P., Jeffrey, P. D.*, et al.* (2000) *Cell* **102**, 533-539
270. Tyers, M., and Willems, A. R. (1999) *Science* **284**, 601 and 603-604
271. Thien, C. B., and Langdon, W. Y. (2001) *Nature Rev. Mol. Cell Biol.* **2**, 294-307
272. Honda, R., and Yasuda, H. (2000) *Oncogene* **19**, 1473-1476
273. Page, A., and Hieter, P. (1999) *Annu. Rev. Biochem.* **68**, 583-609
274. Deshaies, R. J. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 435-467
275. Iwai, K., Yamanaka, K., Kamura, T.*, et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**, 12436-12441
276. Maxwell, P. H., Wiesener, M. S., Chang, G. W.*, et al.* (1999) *Nature* **399**, 271-275
277. Stebbins, C. E., Kaelin, W. G., Jr., and Pavletich, N. P. (1999) *Science* **284**, 455-461
278. Ohh, M., Kim, W. Y., Moslehi, J. J.*, et al.* (2002) *Embo Rep.* **3**, 177-182
279. Patterson, C. (2002) *Sci. STKE* **2002**, PE4
280. Aravind, L., and Koonin, E. V. (2000) *Curr. Biol.* **10**, R132-R134
281. Hatakeyama, S., Yada, M., Matusmoto, M.*, et al.* (2001) *J. Biol. Chem.* **276**, 33111-33120
282. Koegl, M., Hoppe, T., Schlenker, S.*, et al.* (1999) *Cell* **96**, 635-644
283. Johnson, E. S., Ma, P. C. M., Ota, I. M.*, et al.* (1995) *J. Biol. Chem.* **270**, 17442-17456
284. Ballinger, C. A., Connell, P., Wu, Y.*, et al.* (1999) *Mol. Cell Biol.* **19**, 4535-4545
285. Connell, P., Ballinger, C. A., Jiang, J.*, et al.* (2001) *Nature Cell Biol.* **3**, 93-96
286. Meacham, G. C., Patterson, C., Zhang, W.*, et al.* (2001) *Nature Cell. Biol.* **3**, 100-105
287. Wickner, S., Maurizi, M., and Gottesman, S. (1999) *Science* **286**, 1888-1893

288. Demand, J., Alberti, S., Patterson, C., *et al.* (2001) *Curr. Biol.* **11**, 1569-1577
289. Imai, Y., Soda, M., Hatakeyama, S., *et al.* (2002) *Mol. Cell* **10**, 55-67
290. Shimura, H., Hattori, N., Kubo, S., *et al.* (2000) *Nature Genet.* **25**, 302-305
291. Kitada, T., Asakawa, S., Hattori, N., *et al.* (1998) *Nature* **392**, 605-608
292. Imai, Y., Soda, M., Inoue, H., *et al.* (2001) *Cell* **105**, 891-902
293. Baumeister, W., Walz, J., Zuhl, F., *et al.* (1998) *Cell* **92**, 367-380
294. Voges, D., Zwickl, P., and Baumeister, W. (1999) *Annu. Rev. Biochem.* **68**, 1015-1068
295. Kloetzel, P. M. (2001) *Nature Rev. Mol. Cell Biol.* **2**, 179-187
296. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65**, 801-847
297. Glickman, M. H., Rubin, D. M., Coux, O., *et al.* (1998) *Cell* **94**, 615-623
298. Fu, H. Y., Sadis, S., Rubin, D. M., *et al.* (1998) *J. Biol. Chem.* **273**, 1970-1981
299. Groll, M., Bajorek, M., Kohler, A., *et al.* (2000) *Nature Struct. Biol.* **7**, 1062-1067
300. Walters, K. J., Kleijnen, M. F., Goh, A. M., *et al.* (2002) *Biochemistry* **41**, 1767-1777
301. Van Nocker, S., and Vierstra, R. D. (1993) *J. Biol. Chem.* **268**, 24766-24773
302. Hoppe, T., and Jentsch, S. (2000) *Cell* **102**, 577-586
303. Palombella, V. J., Rando, O. J., Goldberg, A. L., *et al.* (1994) *Cell* **78**, 773-785
304. Orian, A., Schwartz, A. L., Israel, A., *et al.* (1999) *Mol. Cell Biol.* **19**, 3664-3673
305. Levitskaya, J., Sharipo, A., Leonchiks, A., *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 12616-12621
306. Murakami, Y. (1992) *Nature* **360**, 597-599
307. Sheaff, R. J., Singer, J. D., Swanger, J., *et al.* (2000) *Mol. Cell* **5**, 403-410
308. Bonifacino, J. S., and Weissman, A. M. (1998) *Annu. Rev. Cell Dev. Biol.* **57**, 1419-1457
309. Brodsky, J. L., and Mccracken, A. A. (1999) *Semin. Cell Dev. Biol.* **10**, 507-513
310. Hicke, L. (1999) *Trends Cell Biol.* **9**, 107-112
311. Lord, J. M., Davey, J., Frigerio, L., *et al.* (2000) *Semin. Cell Dev. Biol.* **11**, 159-164
312. Sommer, T., and Wolf, D. H. (1997) *FASEB Journal* **11**, 1227-1233
313. Kopito, R. R. (1997) *Cell* **88**, 427-430
314. Friedlander, R., Jarosch, E., Urban, J., *et al.* (2000) *Nature Cell Biol.* **2**, 379-384
315. Ward, C. L., Omura, S., and Kopito, R. R. (1995) *Cell* **83**, 121-127.
316. Hampton, R. Y., and Bhakta, H. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 12944-12948
317. Soetens, O., De Craene, J.-O., and Andre, B. (2001) *J. Biol. Chem.* **276**, 43949-43957
318. Helliwell, S., Losko, S., and Kaiser, C. (2001) *J. Cell Biol.* **153**, 649-662
319. Springael, J. Y., Galan, J. M., Haguenauer-Tsapis, R., *et al.* (1999) *J. Cell Sci.* **112**, 1375-1383
320. Springael, J. Y., Nikko, E., Andre, B., *et al.* (2002) *Febs Lett* **517**, 103-109
321. Hicke, L. (1999) *Trends Cell. Biol.* **9**, 107-112
322. Strous, G. J., and Govers, R. (1999) *J. Cell Sci.* **112**, 1417-1423
323. Terrell, J., Shih, S., Dunn, R., *et al.* (1998) *Mol. Cell* **1**, 193-202
324. Lucero, P., Penalver, E., Vela, L., *et al.* (2000) *J. Bacteriol.* **182**, 241-243
325. Galan, J. M., and Haguenauer-Tsapis, R. (1997) *Embo J.* **16**, 5847-5854
326. Springael, J. Y., Galan, J. M., Haguenauertsapis, R., *et al.* (1999) *J Cell Sci* **112**, 1375-1383
327. Mellman, I. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 575-625
328. Shih, S. C., Sloper-Mould, K. E., and Hicke, L. (2000) *Embo J.* **19**, 187-198
329. Dunn, R., and Hicke, L. (2001) *J. Biol. Chem.* **276**, 25974-25981
330. Shenoy, S. K., McDonald, P. H., Kohout, T. A., *et al.* (2001) *Science* **294**, 1307-1313
331. Lin, F. T., Krueger, K. M., Kendall, H. E., *et al.* (1997) *J. Biol. Chem.* **272**, 31051-31057
332. Torrisi, M. R., Lotti, L. V., Belleudi, F., *et al.* (1999) *Mol Biol Cell* **10**, 417-434
333. Drake, M. T., Downs, M. A., and Traub, L. M. (2000) *J. Biol. Chem.* **275**, 6479-6489

334. Riezman, H. (2002) *Nature* **416**, 381-382
335. Gruenberg, J., and Maxfield, F. (1995) *Curr. Opin. Cell. Biol.* **7**, 552-563
336. Hicke, L. (2001) *Cell* **106**, 527-530
337. Futter, C. E., Pearse, A., Hewlett, L. J.*, et al.* (1996) *J. Cell Biol.* **132**, 1011-1023
338. Reggiori, F., and Pelham, H. R. (2001) *Embo J.* **20**, 5176-5186
339. Katzmann, D. J., Babst, M., and Emr, S. D. (2001) *Cell* **106**, 145-155
340. Levkowitz, G., Waterman, H., Zamir, E.*, et al.* (1998) *Gene Develop.* **12**, 3663-3674
341. Levkowitz, G., Waterman, H., Ettenberg, S. A.*, et al.* (1999) *Mol. Cell* **4**, 1029-1040
342. Waterman, H., Levkowitz, G., Alroy, I.*, et al.* (1999) *J. Biol. Chem.* **274**, 22151-22154
343. Longva, K. E., Blystad, F. D., Stang, E.*, et al.* (2002) *J. Cell Biol.* **156**, 843-854
344. VerPlank, L., Bouamr, F., LaGrassa, T. J.*, et al.* (2001) Proc. Natl. Acad. Sci. USA **98**, 7724-7729
345. Bishop, N., and Woodman, P. (2001) *J. Biol. Chem.* **276**, 11735-11742
346. Hofmann, K., and Falquet, L. (2001) *Trends Biochem. Sci.* **26**, 347-350
347. Shih, S. C., Katzmann, D. J., Schnell, J. D.*, et al.* (2002) *Nature Cell. Biol.* **4**, 389-393
348. Bilodeau, P. S., Urbanowski, J. L., Winistorfer, S. C.*, et al.* (2002) *Nature Cell Biol.*
349. Amerik, A. Y., Nowak, J., Swaminathan, S.*, et al.* (2000) *Mol. Biol. Cell* **11**, 3365-3380
350. Babst, M., Katzmann, D., Estepa-Sabal, E.*, et al.* (2002) *Dev. Cell* **3**, 271
351. Babst, M., Katzmann, D., Snyder, W.*, et al.* (2002) *Dev. Cell* **3**, 283
352. Strous, G. J., van Kerkhof, P., Govers, R.*, et al.* (1996) *Embo J.* **15**, 3806-3812
353. van Kerkhof, P., Sachse, M., Klumperman, J.*, et al.* (2001) *J. Biol. Chem.* **276**, 3778-3784
354. van Kerkhof, P., Govers, R., Alves dos Santos, C. M.*, et al.* (2000) *J. Biol. Chem.* **275**, 1575-1580
355. Govers, R., ten Broeke, T., van Kerkhof, P.*, et al.* (1999) *Embo J.* **18**, 28-36
356. Sachse, M., van Kerkhof, P., Strous, G. J.*, et al.* (2001) *J. Cell Sci.* **114**, 3943-3952
357. Govers, R., van Kerkhof, P., Schwartz, A. L.*, et al.* (1998) *J. Biol. Chem.* **273**, 16426-16433
358. van Kerkhof, P., Alves dos Santos, C. M., Sachse, M.*, et al.* (2001) *Mol. Biol. Cell* **12**, 2556-2566
359. Sachse, M., Urbé, S., Oorschot, V.*, et al.* (2002) *Mol. Biol. Cell* **13**, 1313-1328
360. Liu, F. H., Wu, S. J., Hu, S. M.*, et al.* (1999) *J. Biol. Chem.* **274**, 34425-34432
361. Wu, S. J., Liu, F. H., Hu, S. M.*, et al.* (2001) *Biochem. J.* **359**, 419-426
362. Cziepluch, C., Kordes, E., Poirey, R.*, et al.* (1998) *J. Virol.* **72**, 4149-4156
363. Callahan, M. A., Handley, M. A., Lee, Y. H.*, et al.* (1998) *J. Virol.* **72**, 8461
364. Tobaben, S., Thakur, P., Fernandez-Chacon, R.*, et al.* (2001) *Neuron* **31**, 987-999
365. Buchner, E., and Gundersen, C. B. (1997) *Trends Neurosci.* **20**, 223-227
366. Fonte, V., Kapulkin, V., Taft, A.*, et al.* (2002) *Proc. Natl. Acad. Sci. USA* **14**, 9439-44
367. Selkoe, D. J. (2001) *Physiol. Rev.* **81**, 741-766
368. Hirano, T., Kinoshita, N., Morikawa, K.*, et al.* (1990) *Cell* **60**, 319-328
369. Sikorski, R. S., Boguski, M. S., Goebl, M.*, et al.* (1990) *Cell* **60**, 307-317
370. Blatch, G. L., and Lassle, M. (1999) *Bioessays* **21**, 932-939
371. Lamb, J. R., Tugendreich, S., and Hieter, P. (1995) *Trends Biochem. Sci.* **20**, 257-259
372. Das, A. K., Cohen, P. W., and Barford, D. (1998) *Embo J.* **17**, 1192-1199
373. Sudakin, V., Ganoth, D., Dahan, A.*, et al.* (1995) *Mol. Biol. Cell* **6**, 185-197
374. King, R. W., Peters, J. M., Tugendreich, S.*, et al.* (1995) *Cell* **81**, 279-288.
375. Lamb, J. R., Michaud, W. A., Sikorski, R. S.*, et al.* (1994) *Embo J.* **13**, 4321-4328
376. Sikorski, R. S., Michaud, W. A., and Hieter, P. (1993) *Mol. Cell Biol.* **13**, 1212-1221
377. Samejima, I., and Yanagida, M. (1994) *J. Cell Biol.* **127**, 1655-1670

378. Tzamarias, D., and Struhl, K. (1995) *Genes Dev.* **9**, 821-831
379. Dodt, G., Braverman, N., Wong, C., *et al.* (1995) *Nature Genet.* **9**, 115-125
380. Brocard, C., Kragler, F., Simon, M. M., *et al.* (1994) *Biochem. Biophys. Res. Commun.* **204**, 1016-1022
381. Fransen, M., Brees, C., Baumgart, E., *et al.* (1995) *J. Biol. Chem.* **270**, 7731-7736
382. Van der Leij, I., Franse, M. M., Elgersma, Y., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11782-11786
383. Lithgow, T., Glick, B. S., and Schatz, G. (1995) *Trends Biochem. Sci.* **20**, 98-101
384. Moczko, M., Bomer, U., Kubrich, M., *et al.* (1997) *Mol. Cell Biol.* **17**, 6574-6584
385. Banerji, J., Sands, J., Strominger, J. L., *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2374-2378
386. Ozaki, T., Hanaoka, E., Naka, M., *et al.* (1999) *DNA Cell Biol.* **18**, 503-512
387. Manchen, S. T., and Hubberstey, A. V. (2001) *Biochem. Biophys. Res. Commun.* **287**, 1075-1082
388. Thress, K., Evans, E. K., and Kornbluth, S. (1999) *Embo J.* **18**, 5486-5493
389. Thress, K., Song, J., Morimoto, R. I., *et al.* (2001) *Embo J.* **20**, 1033-1041
390. Luders, J., Demand, J., and Hohfeld, J. (2000) *J. Biol. Chem.* **275**, 4613-4617
391. Luders, J., Demand, J., Papp, O., *et al.* (2000) *J. Biol. Chem.* **275**, 14817-14823
392. Takayama, S., Xie, Z., and Reed, J. C. (1999) *J. Biol. Chem.* **274**, 781-786
393. Takayama, S., Bimston, D. N., Matsuzawa, S., *et al.* (1997) *Embo J.* **16**, 4887-4896
394. Zeiner, M., Gebauer, M., and Gehring, U. (1997) *Embo J.* **16**, 5483-5490
395. Takayama, S., and Reed, J. C. (2001) *Nature Cell Biol.* **3**, E237-241
396. Stocker, W., Grams, F., Baumann, U., *et al.* (1995) *Protein Sci* **4**, 823-840
397. Wolfsberg, T. G., and White, J. M. (1996) *Dev. Biol.* **180**, 389-401
398. Black, R. A., and White, J. M. (1998) *Curr. Opin. Cell. Biol.* **10**, 654-659
399. Maskos, K., Fernandez-Catalan, C., Huber, R., *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 3408-3412
400. Yuan, R., Primakoff, P., and Myles, D. G. (1997) *J. Cell Biol.* **137**, 105-112
401. Van Wart, H. E., and Birkedal-Hansen, H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5578-5582
402. Roghani, M., Becherer, J. D., Moss, M. L., *et al.* (1999) *J. Biol. Chem.* **274**, 3531-3540
403. Loechel, F., Overgaard, M. T., Oxvig, C., *et al.* (1999) *J. Biol. Chem.* **274**, 13427-13433
404. Milla, M. E., Leesnitzer, M. A., Moss, M. L., *et al.* (1999) *J. Biol. Chem.* **274**, 30563-30570
405. Itai, T., Tanaka, M., and Nagata, S. (2001) *Eur. J. Biochem.* **268**, 2074-2082
406. Reddy, P., Slack, J. L., Davis, R., *et al.* (2000) *J. Biol. Chem.* **275**, 14608-14614
407. Schlondorff, J., Becherer, J. D., and Blobel, C. P. (2000) *Biochem. J.* **347**, 131-138.
408. Doedens, J. R., and Black, R. A. (2000) *J. Biol. Chem.* **275**, 14598-14607
409. Strous, G., and Gent, J. (2002) FEBS Lett 529, 102

# Chapter II

## Small glutamine-rich tetratricopeptide repeat-containing protein interacts with the ubiquitin-dependent endocytosis motif of the growth hormone receptor

Julia A. Schantl*, Marcel Roza*, Ad P. de Jong†, Ger J. Strous*

*Department of Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands

† Laboratory of Organic Analytical Chemistry, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

*(Manuscript submitted)*

# Abstract

Endocytosis of the growth hormone receptor (GHR) is regulated by the ubiquitin-conjugating system. A cytosolic 10 amino acid motif, referred to as the ubiquitin-dependent endocytosis motif (UbE motif), is involved in the ubiquitination as well as in the endocytosis of the receptor. Proteins that are implicated in one of these processes have not been identified so far. Using a GST-pulldown assay with a GST-fusion protein encompassing the UbE motif of the GHR, a 35 kDa protein was purified. The protein was identified by mass spectrometry as small glutamine-rich tetratricopeptide repeat (TPR) containing protein, SGT. We found that GHR interacts directly with SGT. *In-vivo*, both the precursor and the mature form of the receptor interacted with SGT. Inactivation of the ubiquitin-conjugating system did not affect the GHR-SGT interaction. Binding studies showed that the first TPR motif of SGT interacts with the UbE motif of the GHR. Together these data show that SGT is a GHR interacting protein that binds independent of the ubiquitin-conjugating system.

# Introduction

Growth hormone (GH) regulates a wide range of processes including somatic growth, metabolism, and cellular differentiation, which are mediated by the GH receptor (GHR) [1]. GHR was the first of the class I cytokine receptors to be cloned [2]. Members of this family including receptors for erythropoietin, prolactin, trombopoietin, and several leukins, share a number of structural characteristics [1,3,4]. The extracellular domain contains four paired cysteines and a W-S-X-W-S motif (X representing any amino acid), involved in ligand binding [5]. In the intracellular domain sequence homology is restricted to two motifs, Box1 and Box2. Both motifs are localized to the membrane proximal domain of the cytosolic tail and are known to be involved in signal transduction. Cytokine receptors lack intrinsic kinase activity and share common signaling mechanisms based on the common usage of a group of cytosolic, non-receptor tyrosine kinases of the Janus family (JAK) [1,6]. In the case of the GHR, JAK2 is recruited to Box1 after one GH molecule interacts with two identical GHR molecules. This binding results in the activation of the JAK2 molecules and subsequent tyrosine phosphorylation of JAK2, GHR, and signal transducers and activators of translation (STATs). This leads to a cascade of events initiating signal transduction. In addition, GH induces the activation of the mitogen-activated protein kinase (MAP kinase) and the insulin receptor substrate (IRS) pathway [7].

GH action depends not only on the presence of the hormone in the circulation but also on the availability of its receptor at the cell surface of the target cells. The maintenance of GH binding capacity depends on one hand on the synthesis of new receptor in the ER, receptor processing in the Golgi, and transport to the plasma membrane and on the other hand on the removal of GHR from the cell surface. Two processes are known to contribute to receptor turnover: (1) proteolysis at the cell surface results in the release of the GH binding protein (GHBP), a process referred to as shedding [8] whereas (2) receptor endocytosis leads to the removal of the receptor molecule from the cell surface followed by degradation in the lysosome. Shedding of the GHBP involves the action of the tumor necrosis factor-$\alpha$ converting enzyme (TACE), a transmembrane metalloprotease [9]. Ectodomain shedding of the GHR can be influenced by phorbol esters and growth factors [10].

Endocytosis of the GHR is regulated by the ubiquitin-proteasome system [11], a system best known for its role in the degradation of short lived and abnormal soluble proteins in the cytosol [12]. Recent studies have identified a role of ubiquitination in

endocytosis [13-15]. Ligand binding results in increased ubiquitination of several mammalian receptor proteins, like epidermal growth factor receptor [16], platelet derived growth factor receptor [17], Met receptor [18], *c-kit* receptor [19], T-cell receptor [20] and GH receptor [21]. C-cbl, a tyrosine kinase adaptor protein, is an E3 ubiquitin ligase that mediates EGF receptor ubiquitination and regulates sorting of the receptor into multivesicular bodies, thereby attenuating kinase signaling [22]. For the Met receptor, recent evidence suggests that degradation occurs mainly in lysosomes and that proteasome inhibitors interfere with endocytic trafficking [23]. Only for the GHR it has been shown that endocytosis is mediated by the ubiquitin-proteasome system via a 10 amino acid motif within the GHR cytosolic tail (UbE motif; D-S-W-V-E-F-I-E-L-D) [24]. Mutations of several amino acids within the motif such as phenylalanine at position 327 into alanine abolishes ubiquitination as well as internalization. The UbE motif can thus be considered a specific target for the ubiquitin system.

In this study we searched for proteins that specifically target the UbE motif and asked the question whether they are involved in the ubiquitination and internalization of the GHR. Using GST-fusion proteins encompassing the UbE motif we pulled down a 35 kDa protein, identified by mass spectrometry as small glutamine rich tetratricopeptide repeat-containing protein (SGT). SGT, also known as viral protein U-binding protein (Ubp), was originally discovered as a protein interacting with envelope proteins of two viruses, namely parvorvirus H-1 and human immunodeficiency virus type 1 [25,26]. Sequence analysis of SGT revealed three tandem tetratricopeptide repeats (TPR). TPR motifs are highly degenerated 34 amino acid repeats involved in protein-protein interaction [27]. TPR containing proteins show E3 ligase activity, like CHIP [28], or are able to interact with subunits of the SCF complex, like sgt1 [29]. For SGT it has been shown that the TPR motif is responsible for the interaction with the C-terminal domain of Hsc70 [30]. In an *in-vitro* study SGT negatively influenced the ability of Hsc70 and DnaJ to refold a denatured model protein substrate [31]. Recent studies also indicated a role of SGT in a trimeric protein complex that functions as a synaptic chaperone machinery [32]. SGT was also identified to interact with β-amyloid peptide. Double stranded RNA inhibition results in suppression of toxicity associated with Aβ expression [33]. Our results show that SGT is a GHR-interacting protein both *in-vitro* and *in-vivo* and is interacting UbE motif specific. The activity of the ubiquitin system plays no role in the interaction between SGT and the GHR. SGT binds to the precursor as well as the mature form of the receptor and interacts specifically via its first TPR motif with the UbE motif of the GHR.

# Material and Methods

### *Material*

phSGT/15b expressing hSGT with an N-terminal His-tag in pET-15b was a generous gift of Chung Wang (Institute of Molecular Biology, Academia Sinica, Taipei). phSGT/15b was expressed in *Escherichia coli* (strain BL21) and His-tagged hSGT was purified by Ni-NTA beads (Qiagen) according to the procedure recommended by the manufacturer. Full-length rabbit GHR cDNA in pCB6 was described [21]. Truncated GHR constructs, GHR334 and GHR434, were constructed by introducing a stop codon at the proper positions in the GHR cDNA and were subcloned into pcDNA3.1 (Invitrogen) as previously described [11]. cDNA of internalization deficient mutants $GHR^{F327A}$ and $GHR334^{F327A}$ were constructed by site directed mutagenesis as previously described [11]. Antibody (Mab5) directed against the extracellular domain of the GHR was from AGEN Inc. (Parsippany, NJ). Antibody recognizing hSGT was generated against His-tagged hSGT. Culture media, fetal calf serum (FCS), L-glutamine, and antibiotics for tissue culture were purchased from Gibco.

### *Production of GHR-GST-fusion proteins*

Full length GHR cDNA in pCB6 served as template for the PCR reaction. GHR(271-334) was amplified with primers A and B (Table 1). Primers A and C were used for amplification of $GHR(271\text{-}334)^{F327A}$. PCR was performed using Klentaq polymerase (Clonetech). PCR fragments were cloned into pGEX-3X (Pharmacia) using the restriction enzyme sites placed into the oligonucleotide sequence. The identity of the DNA constructs was confirmed by sequencing. The resulting plasmid was used to express GST-GHR(271-334) and GST-$GHR(271\text{-}334)^{F327A}$ in *E. coli* (strain BL21). The synthesis of recombinant proteins was induced by isopropyl-1-thio-β-D-galactopyranoside. GST and GST-fusion proteins were purified with GSH-beads (Amersham Pharmacia Biotech) by the procedure recommended by the manufacturer.

**Table 1.** Nucleotide sequences of the primers used in this study

| Primers | Nucleotide sequence |
|---|---|
| A | 5′-GATCGGATCCCCAAACAGCAAAGGATTAAGATGCTG |
| B | 5′-GATCGGATCCATGGTCAGTCATCGATGCTAGCTCGATGAA |
| C | 5′-GATCGGATCCATGGTCAGTCATCGATGCTAGCTCGATGCTTCAAC |
| D | 5′-CGGGATCCGGACAACAAGAAGCGCCTGGCCTAC |
| E | 5′-GATCGGATCCTCACTCCTGCTGGTCGTCGTTGC |
| F | 5′-GATCGGATCCTCAGTTGTCGGGGTCCAGCTCCAGAG |
| G | 5′-GATCGGATCCTCAGTAGGCCGGGTCAATGCAGATGG |
| H | 5′-GATCGGATCCTCAGTTGGCTGGGTTGAGCTCGATGG |
| I | 5′-GGATCCAGCAGAGCGCCTCAAAACCGAAGG |
| J | 5′-GGATCCAGCCGTCTATTTCTGCAACAGAGCC |
| K | 5′-GGATCCAGAGACATACAAGTCCAACCTCAAG |

### Production of His-tagged hSGT

phSGT/15b was used to express hSGT in *E. coli* (strain BL21). The synthesis of recombinant proteins was induced by isopropyl-1-thio-β-D-galactopyranoside. hSGT was isolated using Ni-beads (QIAGEN) by the procedure recommended by the manufacturer.

### Generation of hSGT, and hSGT partial constructs

phSGT/15b served as template for the PCR reaction. hSGT full length construct was generated with primers D and E (Table 1). The partial constructs hSGT192, hSGT158, and hSGT124 were generated with primer D as one of the primers and primers F, G, and H as the other primer, respectively. For constructs hSGTΔ90, hSGTΔ124, and hSGTΔ192 primer E was used as one of the primers together with primers I, J, or K, respectively. The PCR-fragments were cloned into pGEM-T easy (Promega) for sequencing, and the inserts ligated into pcDNA3.1/HisB (Invitrogen) or pEF6/HisB (Invitrogen) for expression in mammalian cells.

### Mammalian cells and transfection

HepG2 cells were propagated in DMEM supplemented with 10% FCS, 10 U/ml penicillin and 100 mg/ml streptomycin.

Chinese hamster ts20 cells, bearing a thermolabile ubiquitin-activating enzyme E1 that is inactive at the non-permissive temperature of 41.5°C, were used. Ts20 cells, stably expressing various GHR constructs, were propagated at 30°C with MEMα supplemented with 4.5 g/l glucose, 10% FCS, 10 U/ml penicillin, 100 mg/ml streptomycin and 0.45 mg/ml geneticin. For experiments, cells were grown in 60 mm dishes in the absence of geneticin to a confluency of approximately 80%. Cells received 10 mM sodium butyrate 16 hours before experiments were performed to enhance expression of CMV-driven constructs.

Chinese hamster E36 cells were propagated at 30°C with MEMα supplemented with 4.5 g/l glucose, 10% FCS, 10 U/ml penicillin, and 100 μg/ml streptomycin.

For transient transfection, CHO-ts20 cells and E36 cells were grown to 30% confluency and transfected with GHR334 and GHR334$^{F327A}$ DNA using the calcium phosphate method. 24 hours after transfection the cells were treated with butyrate overnight. Cells were used for experiments 36 hours after transfection.

### Metabolic labeling and subcellular fractionation

For metabolic labeling, HepG2 cells were incubated in methionine-free MEM before [$^{35}$S]methionine (3.7 MBq/ml Trans $^{35}$S Label 40 TBq/mmol, ICN). The incubation was continued for 2 hours. After a 5 min incubation with MEMα cells were swollen in LS buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF) for 15 min. Cells were broken by passing through a 30G needle. Postnuclear supernatant was prepared by centrifugation of homogenates for 5 min at 2000 rpm at 4°C. High speed supernatant and membrane pellet were obtained by centrifugation of PNS at 65.000g for one hour at 4°C in an ultracentrifuge. BSA and Triton X-100 were added to a final concentration of 0.5% (w/v) to the supernatant.

### *In-vitro transcription translation*

cDNA of hSGT or cDNA of SGT partial constructs in pcDNA3.1/HisB 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).

### *In-vitro binding assay*

Equal amount of cytosolic fraction, [$^{35}$S]-labeled proteins from the *in-vitro* transcription translation mixture or isolated hSGT were incubated for 1 hour at 4°C with GST, GST-GHR(271-334) or GST-GHR (271-334)$^{F327A}$ absorbed to GSH beads. Beads were pelleted in an Eppendorf centrifuge and washed three times with 1 ml LS buffer containing 0.5% (w/v) Triton X-100, 0.5% (w/v) BSA and protease inhibitors, and washed twice with PBS containing protease inhibitors. The beads were boiled in 2x sample buffer. Samples were subjected to SDS PAGE or 2D gel electrophoresis. Proteins, associated with the GST-fusion proteins, were visualized by autoradiography of the dried gel or by Coomassie staining.

### *2D gel electrophoresis*

*In-vitro* binding experiment was performed as above. The proteins were resuspended in 350 µl buffer containing 6 M urea, 2 M thiourea, 10 mM dithiothreitol, 2% (w/v) CHAPS, 1% (v/v) Resolytes pH 3.5-10 (BDH), 1% (v/v) Resolytes of pH 4-8 (BDH), and bromphenolblue. For the isoelectric focusing 18 cm Immobiline Dry Strips, pH 3-10, non-linear (Pharmacia) were used. Rehydration of the strips with 320 µl protein samples was performed over night at room temperature. A MultiphorII Electrophoretic Unit (Pharmacia) was used for isoelectric focusing, which was performed at 15°C starting with 300 V. Voltage was increased to 3500 V within 3 hours and continued at 5000 V until 90.000 Vh were reached. The strips were incubated under reducing conditions (6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 25 mM Tris/HCl pH 8.8, 2% (w/v) dithiothreitol) for 20 min and the reaction was stopped for 20 min in an iodacetamide solution (6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 25 mM Tris/HCl pH 8.8, 2.5% (w/v) iodacetamide). Proteins were separated on a 9-16% gradient gel using a Protean II Multi Cell Chamber (Biorad). Proteins were stained with Coomassie. Protein spots were excised and subjected to mass spectrometry.

### *Mass spectrometry*

The protein gel spots of interest were treated with dithiothreitol (DTT) and iodoacetamide and in-gel digested according to Shevchenko *et al.* [35]. The obtained tryptic peptides were analysed by nanoscale liquid chromatography coupled to tandem mass spectrometry [36]. Briefly, peptide mixtures were loaded on to a 50 µm ID x 42 mm precolumn packed with 10 µm C8 particles (Poros 10R1, Perseptive). After loading, the trapped peptides were separated on a 50 µm ID x 206 mm length column packed with 5 µm C18 particles (Purosphere) and subsequently eluted into an ion trap mass spectrometer (LCQ, Finnigan). Fragment ion spectra were recorded in data dependent acquisition mode.

### *Cell lysis, immunoprecipitation and Western blotting*

Cells were lysed on ice in LS buffer containing 0.5% (w/v) Triton X-100, 0.5% (w/v) BSA, and protease inhibitors. The lysates were cleared by centrifugation. Reactions were incubated with GHR or SGT antiserum for 2 hours at 0°C. Protein-A agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immunocomplexes. The immunocomplexes were washed three times with LS buffer containing 0.5% (w/v) Triton X-100, 0.5% (w/v) BSA and protease inhibitors, and washed twice with PBS. Immunocomplexes were analyzed on 4-20% SDS PAGE together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained with either anti-GHR or anti-SGT antibody. After incubating the blots with protein-A conjugated to HRP, antigens were visualized using the ECL system (Boehringer Mannheim).

## Results

### *Identification of binding partners for the UbE motif of GHR*

To search for potential binding partners for the UbE motif of the GHR, involved in the internalization step, we performed GST-pulldown experiments. Therefore, GST or GST-fusion proteins, encompassing the wt UbE motif (GST-GHR(271-334)) or the inactive motif (GST-GHR(271-334)$^{F327A}$) (Fig. 1A), were incubated with the cytosolic fraction of HepG2 cells, metabolically labeled with [$^{35}$S]methionine. The autoradiography showed a 35 kDa protein that was specifically isolated by the GSH beads carrying the UbE motif of GHR, but not by those with GST or the inactive UbE motif (Fig. 1B). We scaled up the purification protocol to obtain Coomassie Blue-stainable amounts of the 35 kDa protein. 2D gel electrophoresis was used to separate the 35 kDa protein from the GST-fusion proteins, which migrated at approximately the same position in the gel. The 35 kDa protein migrated as four individual spots with a pI ranging from 4.4 - 4.9 (Fig. 1C). All four protein-spots were subjected to micro-sequencing by mass spectrometry and were identified as small glutamine-rich tetratricopeptide repeat (TPR) containing protein (SGT). SGT was first identified as a protein putatively interacting with envelope proteins of two viruses, i.e. human immunodeficiency virus type 1 and parvovirus H-1.

In order to investigate whether SGT could interact directly with GHR *in-vitro*, equal amounts of His-tagged SGT expressed in bacteria and isolated with Ni-beads were incubated with GST, GST-GHR(271-334) or GST-GHR(271-334)$^{F327A}$ immobilized on GSH beads. This experiment was performed in the absence or presence of HepG2 cell

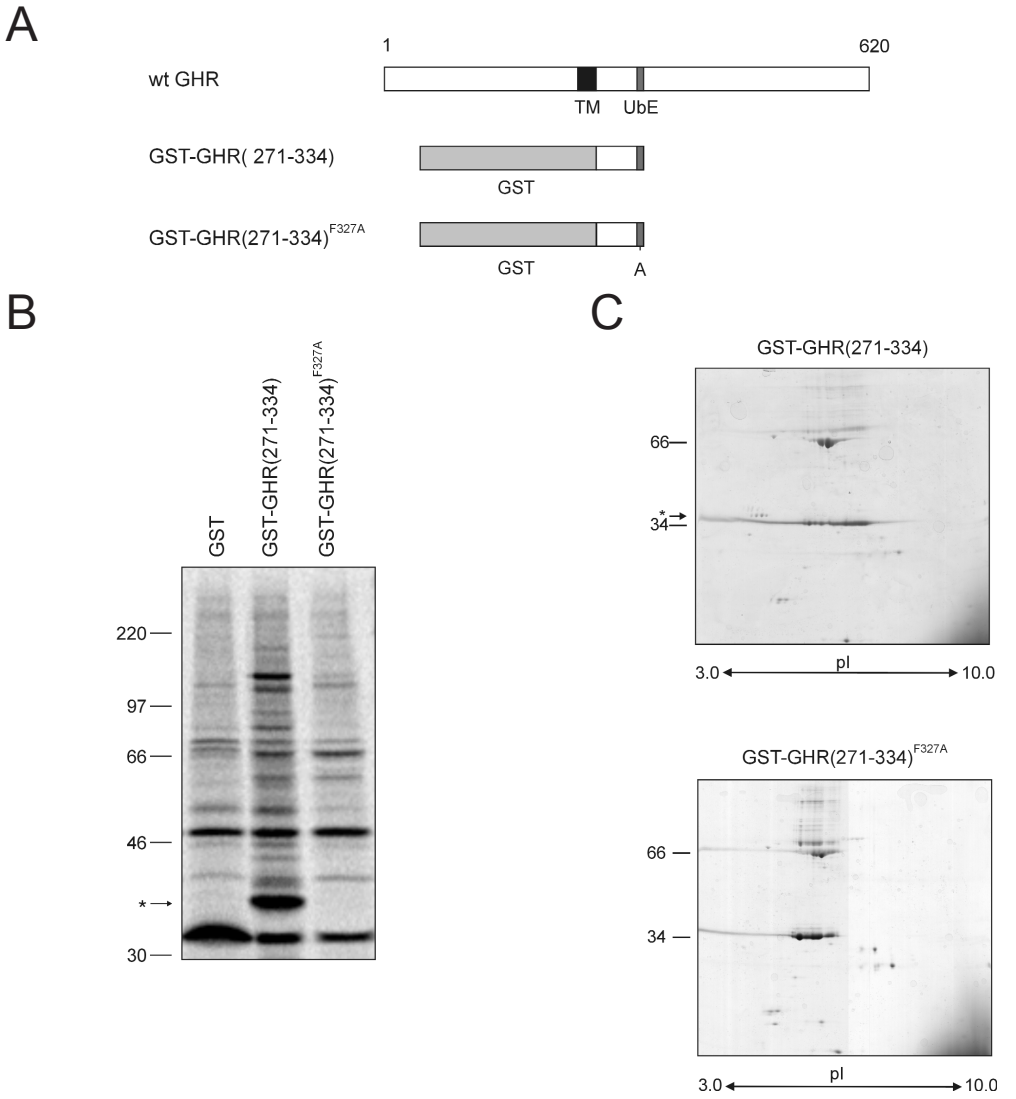



**Figure 1. Isolation of SGT as binding partner of the UbE motif of the GHR.**
**(A)** Schematic representation of the wt GHR and the GST-GHR-fusion proteins used in the pulldown experiments. The black and the gray boxes represent the transmembrane domain (TMD) and the UbE motif (site for interaction with the ubiquitin system), respectively. The light gray box represents GST.
**(B)** HepG2 cells were labeled for 2 hours with [$^{35}$S]methionine and solubilized in 0.5% Triton X-100. Equal amounts of GST or GST-fusion proteins were immobilized on glutathione-Sepharose beads and incubated with equal amounts of cytosol for 1.5 hours. Beads were washed with homogenization buffer before subjecting the amples to SDS page. * indicates the specific 35 kDa band identified.
**(C)** Samples were prepared as described in B and subjecting to 2D gel electrophoresis.

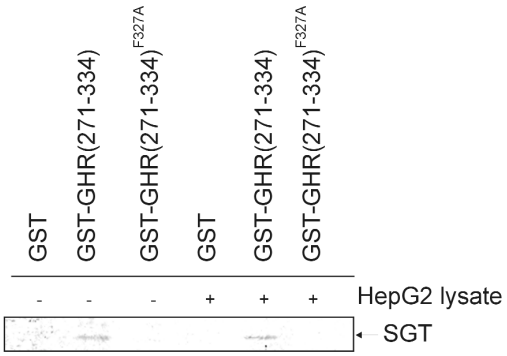



**Figure 2.** ***In-vitro*** **interaction of endogenous SGT with UbE motif of the GHR.**
GST-GHR-fusion proteins and His-tagged SGT expressed in bacteria were incubated either in the absence or the presence of HepG2 lysate for 1.5 hours. Beads were washed with homogenization buffer before subjecting the samples to SDS page. The gel was Coomassie stained.

lysate. As shown in Fig. 2, SGT specifically interacted with GST-GHR(271-334), the fusion protein containing the intact UbE motif. No interaction was observed with GST nor with GST-GHR(271-334)$^{F327A}$. This indicates that the interaction between SGT and the UbE motif of the GHR is direct and specific for the UbE motif. Addition of HepG2 lysate did not result in a significant increase in interaction between SGT and GST-GHR (271-334).

### *Identification of the SGT domain that is involved in the GHR-SGT interaction*

In order to map the UbE motif interacting domain of SGT we constructed several partial constructs of SGT (Fig. 3A). *In-vitro* translated $^{35}$S-labeled SGT constructs were incubated with purified GST, GST-GHR(271-334) or GST-GHR(271-334)$^{F327A}$ bound to GSH beads (Fig. 3B and 3C). As expected, full length SGT showed strong interaction with GST-GHR(271-334), but not with GST-GHR(271-334)$^{F327A}$ or GST alone. Removing the N-terminus (SGTΔ90) or the C-terminus (SGT192) flanking the TPR motifs did not affect the binding of SGT to the UbE motif. When TPR2 and TPR3 were deleted together with the C-terminus, interaction with the UbE motif of the GHR could still be observed whereas no interaction was visible when TPR1 was missing (SGTΔ124, SGTΔ192). This demonstrates that TPR1 is the domain of SGT that directly interacts with the GHR.

### *Characterization of GHR-SGT interaction in-vivo*

We used Chinese hamster ts20 cells stably expressing the wtGHR, GHR434,

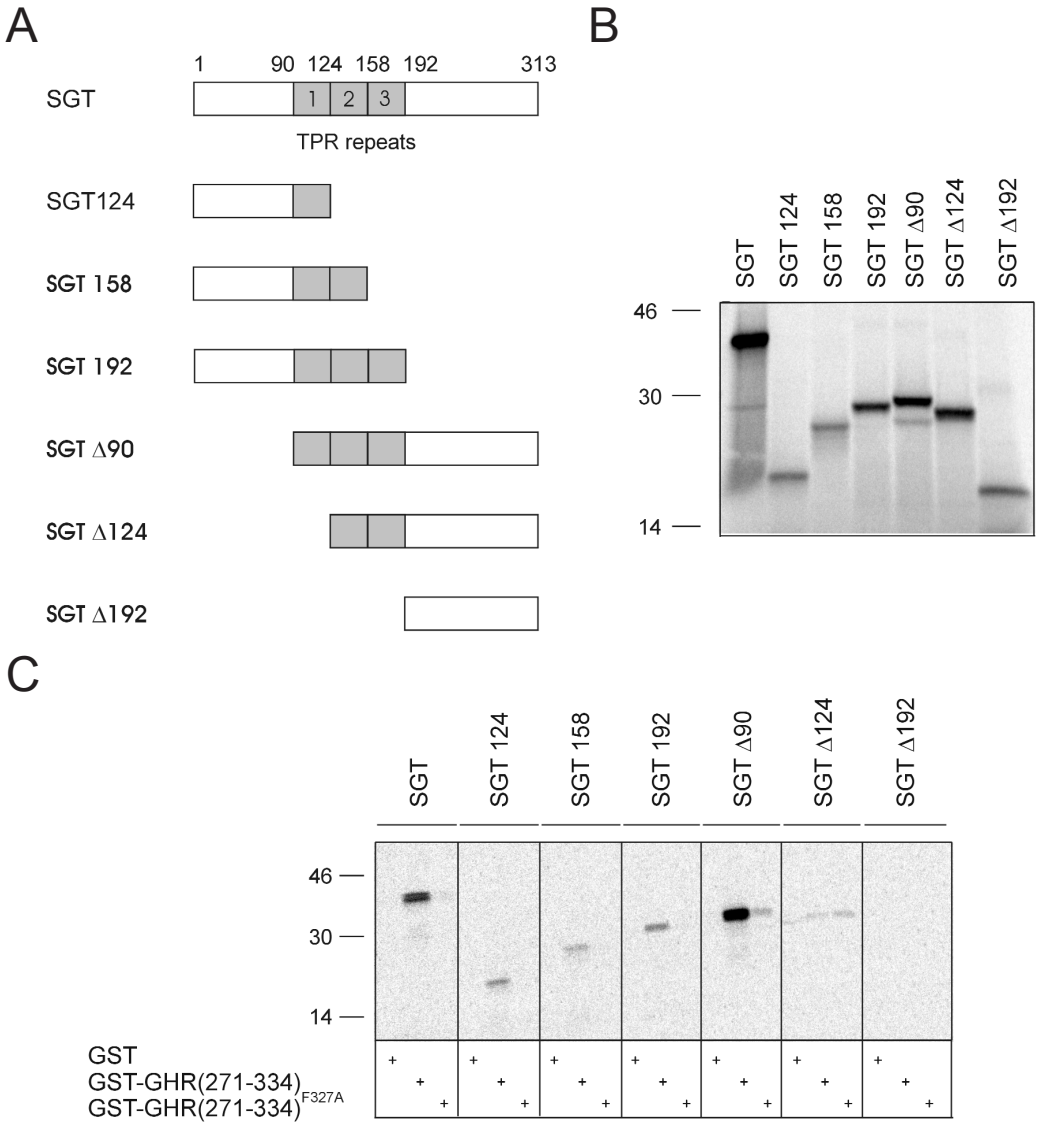



**Figure 3.**
**(A)** Schematic representation of SGT and partial constructs of SGT truncations used in the GST-pulldown experiments. The gray box represents a single TPR-repeat.
**(B)** The different SGT constructs were translated in-vitro in the presence of [$^{35}$S]methionine.
**(C)** Equal amounts of GST or GST-fusion proteins were immobilized on glutathione-Sepharose beads and incubated with equal amounts of in-vitro transcription mix for 1.5h. Samples were subjecting to SDS page.

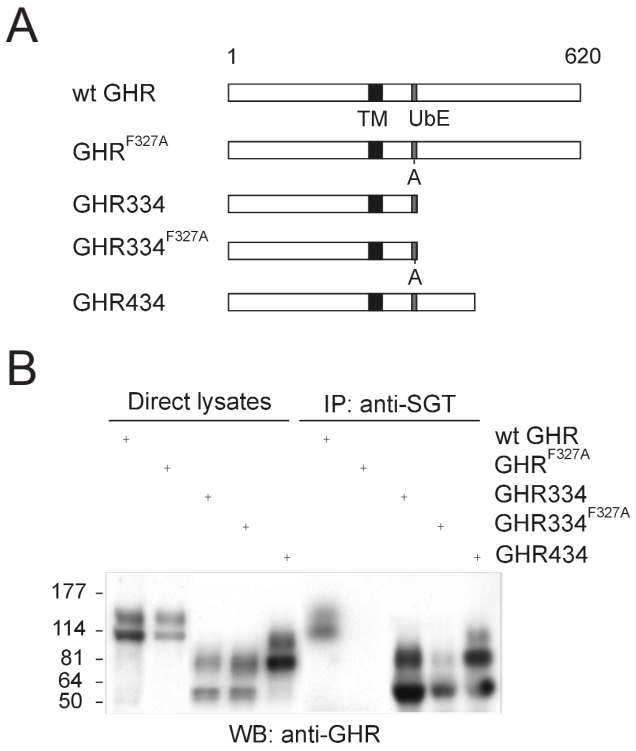



**Figure 4. *In-vivo* interaction between hSGT and GHR.**
**(A)** Schematic representation of the GHR truncations used. The black and the gray boxes represent the transmembrane domain (TMD) and the UbE motif (site for interaction with the ubiquitin system), respectively.
**(B)** Chinese hamster cells stably expressing either wt GHR, GHRPhe327Ala or the respective truncations at amino acid 334 or 434, were lysed in 0.5% Triton X-100. SGT was immunoprecipitated from the cell lysate with anti-SGT serum and analyzed by western blotting with anti-GHR (Mab5).

GHR334 or their corresponding F327A mutant to confirm this GHR-SGT interaction *in-vivo* (Fig. 4A). The cytosolic tail of GHR334 is comparable with the part of the cytosolic tail of GHR that was fused to GST. Immunoprecipitations using an antibody raised against full length SGT resulted in co-immunoprecipitation of the wtGHR as well as of the truncated GHRs. In all cases both the precursor and the mature form of the receptor were immunoprecipitated (Fig. 4B). In the case of the wtGHR less receptor was immunoprecipitated than compared to the truncated receptors. If cell lines expressing the respective mutant receptors were used, no co-immunoprecipitation was observed. The band visible at 55 kDa after co-immunoprecipitation of GHR334$^{F327A}$ does not represent the precursor of the GHR but rather IgG, which was also recognized by the GHR antibody. This band is also visible in the lane of GHR 434 co-immunoprecipitation. For this GHR truncation the precursor form runs at 80 kDa. These results suggest that the interaction of SGT is independent of the length of the cytosolic tail of the GHR and that SGT interacts *in-vivo* with both the precursor and the mature form of the receptor.

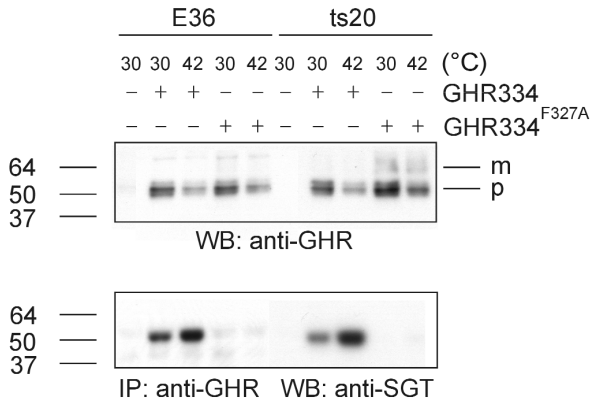



**Figure 5. GHR-hSGT interaction is independent of the ubiqutin system.** Chinese hamster ts20 cells, containing a thermolabile ubiquitin-activating enzyme E1, or Chinese hamster E36 cells were transfected with either GHR334 or GHR334$^{F327A}$. Cells were incubated for 1 hour at 30°C or 41.5°C. After cell lysates were prepared, SGT was immunoprecipitated with anti-SGT serum and samples were analyzed by western blotting with anti-GHR (Mab5). p indicates the precursor GHR (110 kDa), m the mature GHR (130 kDa).

### *Involvement of the ubiquitin system in the SGT-GHR interaction*

As the UbE motif is required for ubiquitin-proteasome system dependent internalization of the GHR, we investigated whether the ubiquitin system plays a role in the interaction between SGT and GHR. We made use of the CHO-ts20 cell line, containing a thermolabile ubiquitin-activating enzyme E1, which is inactive at the non-permissive temperature of 41.5°C. As a control cell line CHO E36 cells were used. Transient transfection of both cell lines with either GHR334 of GHR334$^{F327A}$ resulted in the expression of similar amounts of GHR in both cell lines (Fig. 5, upper panel). Because of the transient transfection the precursor form of the receptor was predominantly produced. After incubating the cells at the permissive or non-permissive temperature, GHR was co-immunoprecipitated with anti-SGT antibody. At 41.5°C the amount of receptor had increased, due to the block of receptor internalization and subsequent degradation. Inactivation of the ubiquitin system did not influence the interaction of SGT with GHR (Fig. 5, lower panel). Due to the accumulation of receptor at 41.5°C the amount of receptor interacting with SGT is increased. These results suggest that the interaction between SGT and the GHR is independent of an intact ubiquitin system and/or that the ubiquitin system acts downstream of SGT.

## Discussion

In this study we identified a potential downstream effector of GHR function called small glutamine-rich tetratricopeptide repeat-containing protein SGT. SGT is able to bind specifically to the UbE motif of the GHR *in-vitro* and *in-vivo*. Four different species

of SGT, which differed in pI, were identified in pulldown experiments with the UbE motif of the GHR. Putative myristoylation, glycosylation, and phosphorylation sites have been postulated for SGT [25]. Regarding the small difference in molecular weight of the isolated SGT species, it is very unlikely that these SGT species are myristoylated or glycosylated. Rather phosphorylation would be the most likely explanation for the difference in pI. It has been shown for other TPR containing proteins that phosphorylation may represent a mechanism by which the interaction with particular proteins can be modulated. Miyata *et al.* have demonstrated that phosphorylation of FKBP52, an other TPR containing protein, by casein kinase II affects the interaction of this protein with Hsp90 [37]. Phosphorylation and dephosphorylation might be involved in the regulation of the SGT-GHR interaction, but as the four SGT species have approximately the same intensity, binding of SGT to the GHR is probably not dependent on this modification. Whether this modification, which does not represent tyrosine phosphorylation (data not shown), plays a role in the interaction with GHR remains to be established. Because SGT contains three TPR motifs, it is tempting to speculate, that these modifications occur in the TPR motifs themselves.

The interaction between the UbE motif of the GHR and SGT takes place through the first TPR motif of SGT. For Δ124 SGT, the construct missing the first TPR motif (TPR1), no interaction with the GHR UbE motif was visible. The two remaining TPR motifs were not able to substitute for the missing TPR motif of SGT, indicating that they might be involved in other protein interactions. Deletion of the first TPR repeat may also result in the disturbance of the structure of the remaining TPR repeats and thereby prevent the interaction between GHR and SGT. For the interaction of SGT with Hsc70 it has been demonstrated that partial removal of the first and the second TPR motif resulted in loss of interaction in a yeast two-hybrid system [30]. This result implies a role either for TPR1, TPR2, or both in the SGT-Hsc70 interaction. Furthermore, SGT might utilize TPR2 and/or TPR3 or the entire TPR domain for its interaction with Hsc70, whereas TPR1 serves as a docking site for the GHR. We cannot exclude yet that SGT can only bind one of the two proteins at the same time via TPR1. Furthermore, we have identified another protein, LRP130, that contains nine pentatricopeptide repeat (PPR) motifs [39], as an UbE motif binding protein (unpublished data). PPR motifs show high sequence similarity with TPR motifs and are predicted to create extended superhelical structures involved in protein-protein interactions [40]. PPR containing proteins are abundant in *Arabidopsis thaliana*, where they localize to mitochondria and peroxisomes. Functionally several of these proteins have been implicated in RNA processing [40].

There are several possibilities for SGT action in GHR function. SGT might function in the process of quality control in the ER, be involved in the transport from the ER to the plasma membrane or be connected to the transport of the receptor from the plasma membrane to the lysosome. From our results it is clear that SGT can interact with the precursor as well as with the mature form of the GHR. This fact gives also some clues as to where the interaction between the GHR and SGT might take place. The precursor form of the receptor resides in the endoplasmic reticulum from where it is transported to the Golgi. Here the protein becomes complex glycosylated. As we can already detect the precursor form of the GHR interacting with SGT, it is quite possible that the interaction already takes place in the ER, possibly as early as during translation of the protein. This interaction could play a role in the correct folding of the GHR as well as in the process of receptor dimerization, which has been shown to occur already in the ER [41]. However, this is unlikely, because there is no defective dimerization in the $GHR^{F327A}$ mutant. The latter does not exclude the possibility that SGT might recognize monomeric receptor molecules and is involved in the removal of monomeric receptors from the ER or maybe from the plasma membrane if they were able to escape quality control in the ER.

Concerning the possible involvement of SGT in the transport of GHR from the ER to the plasma membrane we observed by immunofluorescence that overexpression of a SGT construct missing the N-terminus or the C-terminus of SGT, or the construct missing the N-terminus together with TPR1 did not show an effect on the distribution of the GHR (data not shown). Due to the presence of endogenous SGT we cannot exclude that overexpression of the partial constructs was not high enough to induce a dominant negative effect.

When we examined the effect of overexpressing putative dominant negative constructs on the uptake of GH, we did not observe any effect on the internalization of the GHR-GH complex. Again the dominant negative effect might not be visible due to the high endogenous level of SGT. SGT could play a role in a later step of endocytosis.

The ubiquitin system, which is involved in the internalization of the GHR, does not affect the interaction of SGT with the UbE motif. This indicates that the GHR-SGT interaction might precede the action of the ubiquitin system. At present we cannot exclude the possibility that SGT is interacting with the UbE motif until the ubiquitin system is triggered to interact with the receptor. Thereby SGT might prevent the interaction of the ubiquitin machinery with the receptor on its way from the ER to the cell surface. Future experiments using small RNA-interference might be the method of

choice.

Finally, there is a possible role for SGT in the sorting of the GHR from the endosome to lysosome. This sorting process depends both on an intact UbE motif [42] and the action of the proteasome [43]. As an intact UbE motif is also a prerequisite for SGT interaction, function of SGT in sorting from endosomes to the lysosome cannot be excluded.

The TPR motifs within SGT might give an indication to its function. TPR motifs have been identified in more than 50 functionally unrelated proteins present in organisms as diverse as bacteria and humans. Most TPR-containing proteins are associated with multi-protein complexes, and there is extensive evidence demonstrating that TPR motifs are important in the function of chaperones, cell cycle, transcription, and protein transport complexes. All reports about SGT and a putative function of SGT link the protein to chaperone complexes. Its ability to interact with Hsc70 and to negatively influence the capacity of Hsc70 together with DnaJ to refold an unfolded substrate, indicates a role as a negative regulator of Hsc70 function. CHIP, for example, is a co-chaperone that converts Hsc70 from a protein-folding machine into a degradation factor that functions at the level of the endoplasmic reticulum [28,44,45]. Unlike SGT, CHIP shows E3 ligase activity, which is associated with a U-box at the C-terminus of the protein. In addition, Imai *et al.* reported recently that CHIP can interact directly with Parkin, a RING E3 ligase [46]. By interaction with Parkin CHIP replaces Hsc70 that can bind to the same region of Parkin, and at the same time promotes the ubiquitination activity of Parkin. Another TPR containing protein, sgt1, was identified because of its ability to interact with skp1, a subunit of the SCF E3 ligase complex [29]. For SGT there is no sequence homology to either a U-box sequence or any other sequence that might hint to a function of SGT in ubiquitin ligation. Still it is tempting to speculate that SGT is able to interact with components of the ubiquitin-proteasome system and thereby links the GHR to the ubiquitin-proteasome system. Taken together, SGT might act as a modulator of ubiquitin system controlled downregulation of the GHR by competing for the UbE motif.

## Acknowledgements

We would like to thank Hans Dieplinger and Barbara Schantl for help with 2D electrophoresis and Jürgen Gent, Peter van Kerkhof, and Martin Sachse for stimulating discussions. This work was supported by grants of the Netherlands Organization for

Scientific Research (NWO-902-23-192), and a European Union Network grant (ERBFMRXCT96-0026).

## Abbreviations

CHIP, C-terminus of hsc70-interacting protein; ER, endoplasmic reticulum; GH, growth hormone; GHBP, growth hormone binding protein; GHR, growth hormone receptor; GST, glutathione S-transferase; Hsc, heat-shock cognate protein; Hsp, heat-shock protein; JAK, Janus kinase; LRP130, leucine-rich protein 130 kDa; PPR, pentatricopetide repeat; SGT, small glutamine-rich tetratricopeptide repeat-containing protein; STAT, signal transducers and activators of transcription; TMD, transmembrane domain; TPR, tetratricopeptide repeat; UbE, ubiquitin-dependent endocytosis;

## References

1. Carter-Su, C., Schwartz, J., and Smit, L. S. (1996) *Annu. Rev. Physiol.* **58**, 187-207
2. Waters, M. J., and Friesen, H. (1979) *J. Biol. Chem.* **254**, 6815-6825
3. Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6934-6938
4. Argetsinger, L. S., and Carter-Su, C. (1996) *Physiol. Rev.* **76**, 1089-1107
5. DeVos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255**, 306-312
6. Ihle, J. N., and Kerr, I. M. (1995) *Trends Genet.* **11**, 69-74
7. Herrington, J., and Carter-Su, C. (2001) *Trends Endocrinol. Metab.* **12**, 252-257
8. Baumann, G. (2001) *J. Pediatr. Endocrinol. Metab.* **14**, 355-375
9. Zhang, Y., Jiang, J., Black, R. A., *et al.* (2000) *Endocrinol.* **141**, 4342-4348
10. Alele, J., Jiang, J., Goldsmith, J. F., *et al.* (1998) *Endocrinol.* **139**, 1927-1935
11. Govers, R., van Kerkhof, P., Schwartz, A. L., *et al.* (1997) *Embo J.* **16**, 4851-4858
12. Hershko, A., and Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61**, 761-807
13. Strous, G. J., and Govers, R. (1999) *J. Cell Sci.* **112**, 1417-1423
14. Hicke, L., and Lippincott-Schwartz, J. (2001) *Curr. Opin. Cell. Biol.* **13**, 429-430
15. Dupre, S., Volland, C., and Haguenauer-Tsapis, R. (2001) *Curr. Biol.* **11**, R932-934
16. Galcheva-Gargova, Z., Theroux, S. J., and Davis, R. J. (1995) *Oncogene* **11**, 2649-2655
17. Mori, S., Kanaki, H., Tanaka, K., *et al.* (1995) *Biochem. Biophys. Res. Commun.* **217**, 224-229
18. Jeffers, M., Taylor, G. A., Weidner, K. M., *et al.* (1997) *Mol. Cell. Biol.* **17**, 799-808
19. Miyazawa, K., Toyama, K., Gotoh, A., Hendrie, P.C., *et al.* (1994) *Blood* **83**, 137-145
20. Cenciarelli, C., Hou, D., Hsu, K. C., Rellahan, B. L., *et al.* (1992) *Science* **257**, 795-797
21. Strous, G. J., van Kerkhof, P., Govers, R., *et al.* (1996) *Embo J.* **15**, 3806-3812
22. Levkowitz, G., Waterman, H., Zamir, E., *et al.* (1998) *Gene Develop.* **12**, 3663-3674
23. Hammond, D. E., Urbe, S., Vande Woude, G. F., *et al.* (2001) *Oncogene* **20**, 2761-2770
24. Govers, R., ten Broeke, T., van Kerkhof, P., *et al.* (1999) *Embo J.* **18**, 28-36
25. Ziepluch, C., Kordes, E., Poirey, R., *et al.* (1998) *J. Virol.* **72**, 4149-4156
26. Callahan, M. A., Handley, M. A., Lee, Y. H., *et al.* (1998) *J. Virol.* **72**, 8461
27. Blatch, G. L., and Lassle, M. (1999) *Bioessays* **21**, 932-939

28. Jiang, J., Ballinger, C. A., Wu, Y.*, et al.* (2001) *J. Biol. Chem.* **276**, 42938-42944
29. Kitagawa, K., Skowyra, D., Elledge, S. J.*, et al.* (1999) *Mol. Cell* **4**, 21-33
30. Liu, F. H., Wu, S. J., Hu, S. M.*, et al.* (1999) *J. Biol. Chem.* **274**, 34425-34432
31. Wu, S. J., Liu, F. H., Hu, S. M.*, et al.* (2001) *Biochem J.* **359**, 419-426
32. Tobaben, S., Thakur, P., Fernandez-Chacon, R.*, et al.* (2001) *Neuron* **31**, 987-999
33. Fonte, V., Kapulkin, V., Taft, A.*, et al.* (2002) *Proc. Natl. Acad. Sci. U S A* **99**, 9439-9444
34. van Kerkhof, P., Govers, R., Alves dos Santos, C. M.*, et al.* (2000) *J. Biol. Chem.* **275**, 1575-1580
35. Shevchenko, A., Wilm, M., Vorm, O.*, et al.* (1996) *Anal. Chem.* **68**, 850-858
36. Meiring, H. D., van der Heeft, E., ten Hove, G. J.*, et al.* (2002) *J. Sep. Sci.* **25**, 557-568
37. Miyata, Y., Chambraud, B., Radanyi, C.*, et al.* (1997) *Proc. Natl. Acad. Sci. U S A* **94**, 14500-14505
38. Brinker, A., Scheufler, C., Von Der Mulbe, F.*, et al.* (2002) *J. Biol. Chem.* **277**, 19265-19275
39. Tsuchiya, N., Fukuda, H., Sugimura, T.*, et al.* (2002) *Eur. J. Biochem.* **269**, 2927-2933
40. Small, I. D., and Peeters, N. (2000) *Trends Biochem. Sci.* **25**, 46-47
41. Gent, J., van Kerkhof, P., Roza, M.*, et al.* (2002) *Proc. Nat. Acad. Sci. USA* **99**, 9858-9863
42. Van Kerkhof, P., and Strous, G. J. (2001) *Biochem. Soc. Trans.* **29**, 488-493
43. van Kerkhof, P., Alves dos Santos, C. M., Sachse, M.*, et al.* (2001) *Mol. Biol. Cell* **12**, 2556-2566
44. Cyr, D. M., Hohfeld, J., and Patterson, C. (2002) *Trends Biochem. Sci.* **27**, 368-375
45. Hohfeld, J., Cyr, D. M., and Patterson, C. (2001) *Embo Rep.* **2**, 885-890
46. Imai, Y., Soda, M., Hatakeyama, S.*, et al.* (2002) *Mol. Cell* **10**, 55-67

# Chapter III

## The anti-apoptotic protein Scythe interacts with the ubiquitin-dependent endocytosis motif of the growth hormone receptor and localizes to mitochondria

Julia A. Schantl*, Marcel Roza*, Ad P. de Jong†, Ger J. Strous*

*Department of Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands

† Laboratory of Organic Analytical Chemistry, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

*(Manuscript in preparation)*

# Abstract

The effects of growth hormone (GH) on its target cells are mediated via the growth hormone receptor (GHR). GH binding to its receptor results in the formation of a $GHR_2$-GH complex and initiates signal transduction cascades through the activation of the tyrosine kinase JAK2. Subsequently, the ligand-receptor complex is endocytosed and transported to the lysosome, where it is degraded. The ubiquitin system regulates both endocytosis and transport to the lysosomes. A cytosolic 10 amino acid motif, designated as the ubiquitin-dependent endocytosis motif (UbE motif), is involved in the ubiquitination as well as in the endocytosis of the receptor. Proteins that are implicated in one of these processes have not been identified so far. Using a GST-pulldown assay with a GST-fusion protein encompassing the UbE motif of the GHR, a 150 kDa protein, identified as Scythe, was purified. Human Scythe (hScythe) has been implicated in the control of apoptosis and regulation of Hsc70 activity. We found that endogenous hScythe localized both to mitochondria and to the nucleus; in addition, hScythe was also present in the Golgi area. The localization to mitochondria showed only a partial overlap, indicating that hScythe resides on specialized subdomains of the mitochondria. Induction of apoptosis with UV did not influence the nuclear localization of hScythe, whereas the mitochondrial labeling was reduced due to a loss of the organelle after the UV treatment. Treatment with cycloheximide resulted in a loss of mitochondrial localization. At the same time labeling in the Golgi area and the nucleus remained unchanged. These results provide evidence of an anti-apoptotic protein, hScythe, which interacts with the GHR, and localizes to mitochondrial structures, where it might interact with elements of the apoptotic machinery.

# Introduction

Human growth hormone (hGH) is a 191 amino acid polypeptide that is synthesized and secreted primarily by somatotrophic cells in the anterior pituitary [1]. Secretion of GH is pulsatile and regulated by two hypothalamic hormones: growth hormone releasing hormone (GHRH), which stimulates secretion, and somatostatin, which inhibits secretion [2]. GH synthesis in a number of extra-pituitary tissues has been reported, suggesting a local autocrine/paracrine way of action [3]. One of the major functions of GH is to stimulate growth of bone and soft tissue. In addition, GH regulates protein, lipid and carbohydrate metabolism throughout life [1,4] and acts as a survival factor [5].

The multiple actions of GH are initiated when the hormone binds to the growth hormone receptor (GHR), a 620 amino acid type I transmembrane glycoprotein. GHR is ubiquitously expressed throughout the body with high expression levels in liver and adipose tissue [6,7]. Based on structural and amino acid homologies, GHR has been classified as a member of the cytokine/hematopoietin receptor superfamily [7]. Members of this receptor family share the lack of intrinsic kinase activity. Binding of GH induces the recruitment and activation of the tyrosine kinase JAK2 [8]. The activated JAK2, in turn, phosphorylates GHR, and both the phosphorylated receptor and JAK2 provide docking sites for a variety of signaling molecules that contain Src homology 2 (SH2) or other phosphotyrosine binding (PTB) motifs, thereby activating specific signaling pathways [9]. Among the activated proteins are the signal transducers and activators of transcription (STATs) [10], which regulate the transcription of GH responsive genes like c-fos [11] and serine protease inhibitor 2.1 [12]. GH can also activate the mitogen-activated protein kinase (MAP kinase) pathway, which is involved in the regulation of gene transcription, cellular growth, and differentiation [13]. GH signaling also activates insulin-receptor substrate (IRS) proteins, IRS-1 and IRS-2, which regulate glucose transport and lipid synthesis [14]. In addition, GH-induced proliferation is regulated via the phosphatidylinositol-3-kinase (PI-3-kinase)/Akt pathway, which is in part responsible for the activation of nuclear factor-κB (NF-κB) [15]. NF-κB activation by GH is critical for cell survival in pro-B Ba/F3 cells [16].

The GHR is constitutively internalized via clathrin-coated pits and subsequently transported via endosomes to lysosomes, where degradation occurs [17-19]. The number of GHR at the cell surface does neither depend on GH in the circulation nor on its signaling, but is independently regulated by the ubiquitin system [20]. Endocytosis and sorting to the lysosome both depend on a ten amino acid motif including phenylalanine-

327 in the cytosolic tail of the receptor, termed the ubiquitin-dependent endocytosis motif (UbE motif, DSWVEFIELD) [21]. GHR ubiquitination occurs at the plasma membrane and coincides with the recruitment of the receptor into clathrin-coated pits [19,22]. However, ubiquitination of the receptor itself is not necessary for ligand internalization [21]. Mutation of phenylalanine at position 327 into alanine results in an internalization incompetent receptor and prevents ubiquitination of the receptor [18]. The UbE motif is also essential for effective sorting to the lysosome, shown by the fact that a truncated GHR, which internalizes ubiquitin system independent, still requires an intact UbE motif for sorting to the lysosome [23].

In this study we searched for proteins that specifically target the UbE motif. Using GST-fusion proteins encompassing the UbE motif we pulled down a 150-kDa protein, identified by mass spectrometry as human Scythe (hScythe). hScythe, previously termed Bat3 (for HLA-B associated transcript 3), was originally identified as one of the genes located within the human major histocompatibility complex (MHC) [24]. Subsequently, rat Bat3 was isolated and shown to be expressed in most adult tissues with high levels in testis. Scythe possesses an N-terminal ubiquitin-like domain (UBD), a central proline rich region, and a zinc-finger like domain [24]. The ubiquitin-like domain shows 37% identity and 54% similarity to the human ubiquitin protein [24]. More recently, two additional functional domains have been identified. A nuclear localization signal in the C-terminus of Scythe was described to be responsible for a nuclear localization of hScythe [25], even though Ozaki *et al.* report that rat Bat3 shows cytosolic localization [26]. Furthermore, Scythe possesses a conserved C-terminal BAG-domain [27], which is a roughly 50 amino acid evolutionary conserved domain with homology to a domain in BAG-1 family members [28,29]. The BAG-domain is involved in the interaction with the ATPase domain of Hsp70 and the regulation of Hsp70 activity. Indeed, in a yeast two-hybrid screen hScythe interacts with the ATPase domain of human Stch, a protein that is similar to Hsp70 [30]. In addition, *Xenopus* Scythe (xScythe) inhibits Hsp70 mediated protein folding activity [27]. *Drosophila* Reaper, a potent apoptotic regulator, can reverse the inhibitory effect of xScythe on Hsp70 [27]. Thress *et al.* have shown that xScythe interacts *in-vitro* with *Drosophila* Reaper [31], and that the presence of xScythe is critical for Reaper-induced apoptosis in *Xenopus* egg extracts [32]. Although Reaper homologues have not been identified in other systems, *Drosophila* Reaper is capable to induce apoptosis in mammalian cells by triggering cytochrome c release and caspase activation [33]. The current model suggests that Scythe sequesters a positive regulator of apoptosis that, when not bound by Scythe, can trigger cytochrome c release from purified

mitochondria in the absence of other cytosolic components; binding of Reaper to Scythe will then liberate this cytochrome c releasing activity [32].

Our results demonstrate that hScythe can interact with the GHR UbE motif *in-vitro*. With an antibody raised against the C-terminus of hScythe, we were able to demonstrate that endogenous hScythe localized in the nucleus, in mitochondria, and perinuclear Golgi structures in HeLa cells. Induction of apoptosis by UVC radiation left nuclear hScythe unchanged. Mitochondrial structures were barely visible and showed no colocalization with hScythe. Treatment with proteasome inhibitor MG132 did not alter hScythe localization, while cyclohexamide showed a reduction of hScythe localizing to mitochondria.

# Material and Methods

### *Material*

Bat3 cDNA in pSK(-) was a generous gift of Sally Kornbluth (Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA). Full-length rabbit GHR cDNA in pCB6 has been described [34]. Antibody, recognizing the cytoplasmic domain of the GHR, was raised against amino acids 271-318 of the cytosolic tail of the GHR [35]. Antibody (Mab5) directed against the extracellular domain of the GHR was from AGEN Inc. (Parsippany, NJ). Antibody recognizing hScythe was generated against GST-hScythe(892-1126). MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was purchased from Calbiochem-Novabiochem, cycloheximide was purchased from ING Biomedicals Inc. Culture media, fetal calf serum (FCS), L-glutamine, and antibiotics for tissue culture were purchased from Gibco.

### *Production of GHR-GST-fusion proteins*

Full length GHR cDNA in pCB6 [34] served as template for the PCR reaction. GHR(271-334) was amplified with primers A and B (Table 1). Primers A and C were used for amplification of GHR(271-334)$^{F327A}$. PCR was performed using Klentaq polymerase (Clonetech). PCR fragments were cloned into pGEX-3X (Pharmacia) using the restriction enzyme sites placed into the oligonucleotide sequence. The identity of the DNA constructs was confirmed by sequencing. The resulting plasmid was used to express GST-GHR(271-334) and GST-GHR(271-334)$^{F327A}$ in *Escherichia coli* (strain BL21). The synthesis of recombinant proteins was induced by isopropyl-1-thio-β-D-galactopyranoside. GST and GST-fusion proteins were purified with GSH-beads (Amersham Pharmacia Biotech) by the procedure recommended by the manufacturer.

### *Production of hScythe-GST-fusion protein*

Bat3 cDNA in pSK(-) served as template for the PCR reaction. hScythe(892-1126) was amplified with primers D and E (Table 1). PCR fragments were cloned into pGEX-5X-3

**Table 1.** Nucleotide sequences of the primers used in this study

| Primers | Nucleotide sequence |
|---|---|
| A | 5′-GATCGGATCCCCAAACAGCAAAGGATTAAGATGCTG |
| B | 5′-GATCGGATCCATGGTCAGTCATCGATGCTAGCTCGATGAA |
| C | 5′-GATCGGATCCATGGTCAGTCATCGATGCTAGCTCGATGCTTCAAC |
| D | 5′-TCTAGACTAAGGCTAATCAGCAAAGGCCC |
| E | 5′-GAATTCCGTGAAACCGCAGCCCCCTCTGAG |

(Pharmacia) using the restriction enzyme sites placed into the oligonucleotide sequence. The identity of the DNA constructs was confirmed by sequencing. The resulting plasmid was used to express GST-hScythe(892-1126) in *E. coli* (strain BL21). 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.

### *Mammalian cells*

HepG2 cells were propagated at 37ºC in DMEM supplemented with 10% fetal calf serum, 10 U/ml penicillin, and 100 mg/ml streptomycin. For experiments, cells were grown in 150 mm dishes to a confluency of 80%.

HeLa cells were propagated in DMEM supplemented with 10% fetal calf serum, 10 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. For experiments, cells were grown in 60 mm dishes to a confluency of 80%.

### *Metabolic labeling and cell lysis*

For metabolic labeling, HepG2 cells were incubated in methionine-free MEM before [$^{35}$S]methionine (3.7 MBq/ml Trans $^{35}$S Label 40 TbQ/mmol, ICN, Costa Mesa, CA). The incubation was continued for 2 hours followed by a 5 min incubation with MEMα. Cells were lysed on ice in lysis buffer (10 mM Tris HCl pH 7.4, 0.5 (w/v)% BSA, 0.5 (v/v)% Triton X-100, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF) for 10 min. The lysates were cleared by centrifugation for 5 min at 2000 rpm at 4°C.

### *In-vitro binding assay*

Equal amounts of cell lysates were incubated for 1 hour at 4°C with GST, GST-GHR(271-334) or GST-GHR (271-334)$^{F327A}$ absorbed 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 of the dried gel or transferred to polyvinylidene difluoride paper for immunoblotting with anti-Scythe antibody. After incubating the blots with protein A conjugated to HRP, antigens were visualized using the ECL system (Boehringer Mannheim).

### Mass spectrometry

The protein gel spots of interest were treated with dithiothreitol (DTT) and iodoacetamide and in-gel digested according to Shevchenko *et al.* [36]. The tryptic peptides obtained were analyzed by nanoscale liquid chromatography coupled to tandem mass spectrometry [37]. Briefly, peptide mixtures were loaded on to a 50 µm ID x 42 mm precolumn packed with 10 µm C8 particles (Poros 10R1, Perseptive). After loading, the trapped peptides were separated on a 50 µm ID x 206 mm length column packed with 5 µm C18 particles (Purosphere) and subsequently eluted into an ion trap mass spectrometer (LCQ, Finnigan). Fragment ion spectra were recorded in data dependent acquisition mode.

### UVC treatment

HeLa cells were grown on coverslips in 12-wells dishes. Before UVC radiation cells were incubated for 20 hours in DMEM supplemented with 0.075% FCS [38]. Medium was removed, and UV treatment was performed in a Stratalinker (Stratagene) with a dose of 400 J/m$^2$. After treatment with UV light, 1 ml of serum-free medium was added and cells were incubated at 37°C. 120 min after UV treatment 1 mM MitoTracker Red (Molecular Probes) was added to the medium. Before fixation, random field photographs were taken, and cell death was morphologically scored as percentage of cells that showed cell blebbing or shrinkage of cells. Cells were fixed at room temperature with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and permeabilized for 5 min with 1% Triton X-100 in PBS. Endogenous Scythe was labeled with polyclonal anti-Scythe followed by Alexa 488-conjugated goat-anti-rabbit IgG (Molecular Probes). Coverslips were embedded in Moviol containing 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) and studied by confocal scanning laser microscopy using a Leica TCS 4D system.

### Microscopy

HeLa cells were grown on coverslips and treated with 20 µM MG132 or 350 µM cycloheximide for 3 hours. 30 min before fixation 1 mM MitoTracker Red (Molecular Probes) was added to the medium. Cells were washed with PBS and fixed for 60 min at room temperature with 4% paraformaledyde in 0.1 M sodium phosphate buffer, pH 7.4. Cells were permeabilized for 5 min with 1% Triton X-100 in PBS. Endogenous Scythe was labeled with polyclonal anti-Scythe followed by Alexa 488-conjugated goat-anti-rabbit IgG (Molecular Probes). Coverslips were embedded in Moviol containing 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) and studied by confocal scanning laser microscopy using a Leica TCS 4D system.

## Results and Discussion

### Identification of binding partners for the UbE motif of GHR

To search for potential binding partners for the UbE motif of the GHR, we performed GST-pulldown experiments. Therefore, GST or GST-fusion proteins,

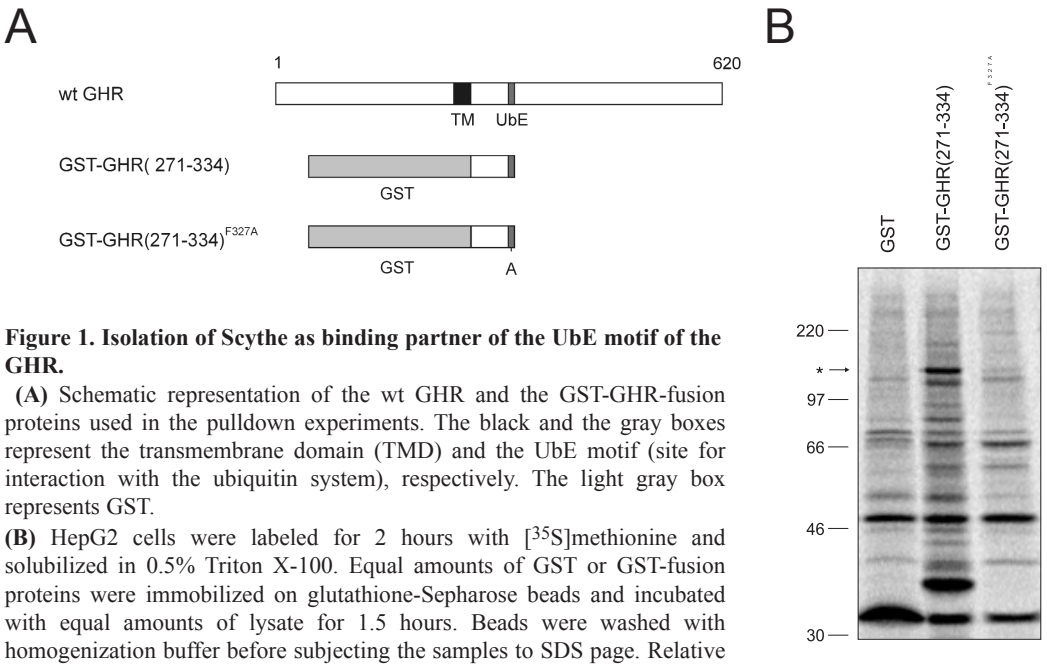



**Figure 1. Isolation of Scythe as binding partner of the UbE motif of the GHR.**

**(A)** Schematic representation of the wt GHR and the GST-GHR-fusion proteins used in the pulldown experiments. The black and the gray boxes represent the transmembrane domain (TMD) and the UbE motif (site for interaction with the ubiquitin system), respectively. The light gray box represents GST.

**(B)** HepG2 cells were labeled for 2 hours with [$^{35}$S]methionine and solubilized in 0.5% Triton X-100. Equal amounts of GST or GST-fusion proteins were immobilized on glutathione-Sepharose beads and incubated with equal amounts of lysate for 1.5 hours. Beads were washed with homogenization buffer before subjecting the samples to SDS page. Relative molecular weight standards (Mr x 10-3) are shown at the left. * indicates the specific 150 kDa band identified.

encompassing the wild type UbE motif (GST-GHR(271-334)) or the inactive motif (GST-GHR(271-334)$^{F327A}$) (Fig. 1A), were incubated with cell lysates of HepG2 cells, metabolically labeled with [$^{35}$S]methionine. The autoradiography showed a 150-kDa protein that was specifically isolated by the GSH beads carrying the UbE motif of GHR, but not by those with GST or the inactive UbE motif (Fig. 1B). We scaled up the purification protocol to obtain Coomassie stainable amounts of the 150-kDa protein. The 150-kDa band was subjected to micro-sequencing by mass spectrometry and was identified as Bat3 (for HLA-B associated transcript 3), now referred to as hScythe. A remarkable feature of this protein is its N-terminal ubiquitin-like domain (UBD). Furthermore, hScythe interacts with Hsp70 and negatively regulates the folding activity of Hsp70 [27]. Reaper, a potent inducer of apoptosis, is another binding partner of hScythe. hScythe itself shows anti-apoptotic properties [31,32].

For further experiments we raised an antibody against amino acids 849-1126 of hScythe. The antibody specifically recognized a protein of 150 kDa in HeLa cell lysate (Fig. 2, first lane) that was not detected with pre-immune serum (not shown). To confirm

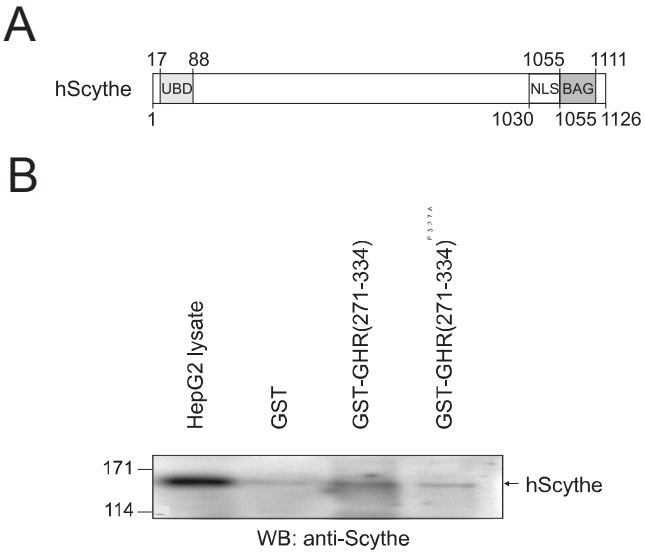



**Figure 2. *In-vitro* interaction of endogenous hScythe with the UbE motif of the GHR.**
**(A)** Schematic representation of hScythe. The light gray, dark gray, and the white box represent the ubiquitin-like domain (UBD), the BAG domain and the nuclear localization signal (NLS), respectively.
**(B)** GST-GHR-fusion proteins and HeLa cell extract were incubated for 1.5 hours. Beads were washed with homogenization buffer before subjecting the samples to SDS page. Immunoblotting were performed with anti-hScythe antibody. Relative Molecular weight standards (Mr x 10-3) are shown at the left. WB, Western blot detection.

the data from the initial pulldown experiments, we repeated the experiment and detected it with the anti-Scythe antibodies. Again, the interaction between GST-GHR(271-334) depended on an intact UbE motif of the GHR, whereas GST and GST-GHR(271-334)$^{F327A}$ showed only some unspecific interaction (Fig. 2). This indicates that the *in-vitro* interaction between hScythe and the GHR is specific for the UbE motif.

These results show the identification of a potential downstream effector of GHR function called hScythe. hScythe, an anti-apoptotic protein, is able to bind specifically to the UbE motif of the GHR *in-vitro.* Due to high unspecific binding of hScythe to IgG we were unable to reproduce these data *in vivo*, nor could we identify the domain that is responsible for this interaction. Therefore, we cannot conclude about the nature of interaction between GHR and hScythe, nor about the function hScythe might have in GHR function. Nevertheless, the finding that an anti-apoptotic protein interacts with the GHR *in-vitro* is interesting because evidence is accumulating that GH is able to inhibit apoptosis in diverse cell systems [39,40].

### *Localization of endogenous hScythe in HeLa cells*

To obtain more insight in the general function of hScythe, we investigated the cellular localization of endogenous hScythe in HeLa cells using the anti-hScythe antibody. Recent studies have been performed only with transiently transfected hScythe.

This led to the description of a nuclear localization of HA-tagged full-length hScythe in HeLa cells [25], whereas green fluorescent tagged rat Bat3 was described to localize mostly in the cytoplasm of COS cells [26]. Endogenous hScythe, however, labeled long strings throughout the cytosol, the perinuclear area, and the nucleus (Fig. 3A). Labeling of the nucleus was visible but not the predominant location of hScythe in the cell. The string-like structures in the cytosol showed high similarity seen when labeling mitochondria. To confirm the localization to mitochondria, we treated the cells with MitoTracker Red for 30 min before fixation. MitoTracker labeled the same string-like structures, indicating that hScythe localized to mitochondrial structures (Fig. 3A-C). However, the overlap was not complete, because hScythe localized to distinct subdomains of mitochondria. Furthermore, the perinuclear structure showed no overlap with MitoTracker Red. To further characterize this perinuclear compartment, cells were double labeled for GM130 and hScythe. GM130 is known to label the cis-Golgi compartment and clearly co-localized with perinuclear hScythe in HeLa cells, confirming that hScythe can localize to the Golgi complex (Fig. 3D–F).

Mitochondria play an important role in the induction of apoptosis via release of pro-apoptotic agents and/or disruption of cellular energy metabolism. Cytochrome c was the first characterized mitochondrial factor shown to be released from the mitochondrial intermembrane space and to be actively implicated in apoptotic cell death. Since then, other mitochondrial proteins, such as AIF, Smac/DIABLO, endonuclease G and Omi/HtrA2, were found to undergo release during apoptosis and have been implicated in various aspects of the cell death process. Members of the Bcl-2 protein family control the integrity and response of mitochondria to apoptotic signals. The finding that hScythe localized to mitochondrial structures is provocative, because it is believed that hScythe sequesters a so far unknown pro-apoptotic protein, which, if not bound to hScythe, induces cytochrome c release from mitochondria [32]. Release of this pro-apoptotic factor is initiated by binding of Reaper to Scythe. Reaper is a pro-apoptotic protein that localizes to punctate perinuclear structures that are also positive for inhibitors of apoptosis (IAPs) [41]. It is tempting to speculate that the perinuclear Golgi region to which hScythe localized could be the same structure like the structure to which Reaper and IAPs localize.

To investigate whether Scythe localization depends on the induction of apoptosis, we induced apoptosis by UVC radiation. After over night serum starvation, cells were treated with UVC light and analyzed for apoptosis by morphological criteria, like cell shrinkage, rounding of cells and cell blebbing. Control cells that received no UV

radiation showed less than 20% apoptotic cells, which were due to serum deprivation for 20 hours (Fig. 4A). Cells that were treated with UVC radiation showed more than 90% apoptotic cell structures 150 min after UV treatment. (Fig. 4 A). Apoptotic cells were incubated with MitoTracker Red before fixation. Cells were stained with antibody recognizing endogenous Scythe. Cells had shrunk and rounded after treatment with UV radiation and clearly showed blebbing at the cell surface (Fig. 4B). Induction of apoptosis did not change the nuclear localization of hScythe. However, mitochondrial structures were not or hardly visibly after treatment with UV radiation. This might indicate that as a result of the UV radiation the integrity of mitochondria has been destroyed resulting in a loss of mitochondrial matrix [42]. The labeling for mitochondria still visible did not show any co-localization with hScythe (Fig. 4B). Scythe labeling was still visible in the cytoplasm, and might represent Golgi labeling. The finding that the nuclear staining remains unchanged after induction of apoptosis is in agreement with the results of Manchen *et al.*, who reported unchanged nuclear localization of hScythe in apoptotic cells after 3 hours staurosporine treatment [25].

hScythe has also been implicated in negative regulation of protein folding, a process that has been closely linked with protein degradation by the proteasome, as for example in the case of BAG-1. Like BAG-1, hScythe contains a ubiquitin-like domain at the N-terminus, which might be involved in protein binding. Another analogy with BAG-1 is the presence of a BAG domain that is responsible for binding to Hsp70. Protein folding, translocation, and degradation are important processes in response to cell stress, proliferation and apoptosis. Therefore, we investigated the effect of a three hour treatment with either cycloheximide or proteasome inhibitor MG132 on hScythe localization. Inhibition of protein degradation by MG132 left hScythe localization unchanged (Fig. 5A). Cycloheximide treatment resulted in a remarkable reduction of hScythe labeling to the mitochondria, whereas the labeling of hScythe in the nuclear or Golgi region remained unchanged (Fig. 5A). This implies that there are two pools of hScythe, a nuclear/Golgi pool and a mitochondrial pool that showed different behavior in the presence of cyclohexamide. Blotting experiments of cycloheximide treated cells did not show a decrease of total hScythe, protein levels rather remained stable over time. There are two possible explainations: (1) the mitochondrial pool has a high turnover as would be expected for a protein complexed with Reaper and IAPs, while the nuclear pool is stable and stationary. (2) The induction of apoptosis induces redistribution of hScythe to the nucleus, which would explain the stability of hScythe on the Western blot. (Fig. 5B).

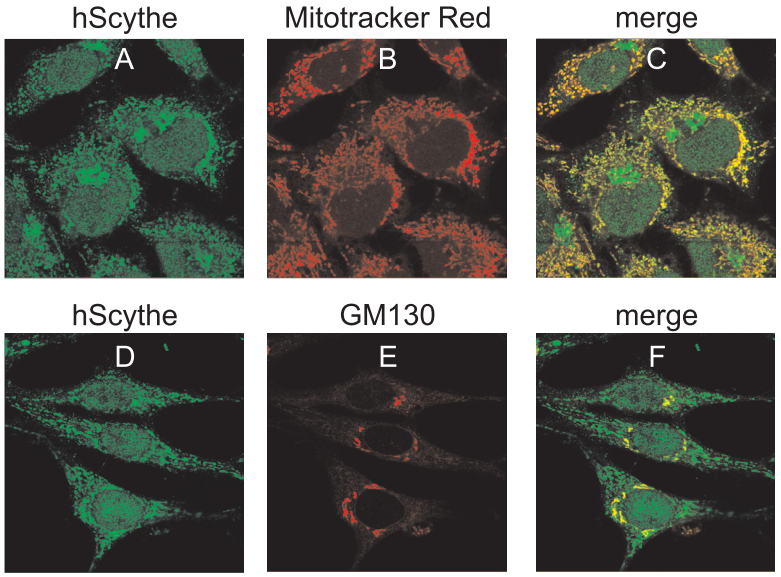



**Figure 3. Subcellular localization of endogenous hScythe.** Cells were incubated for 30 min with MitoTracker Red (B), fixed, permeabilized, and stained with anti-hScythe antibody (A and D) and anti-GM130 antibody (E). Detection was facilitated using an Alexa 488 conjugated secondary antibody and visualized using confocal laser scanning microscopy. Co-localization is visualized in the merged images (C and F).

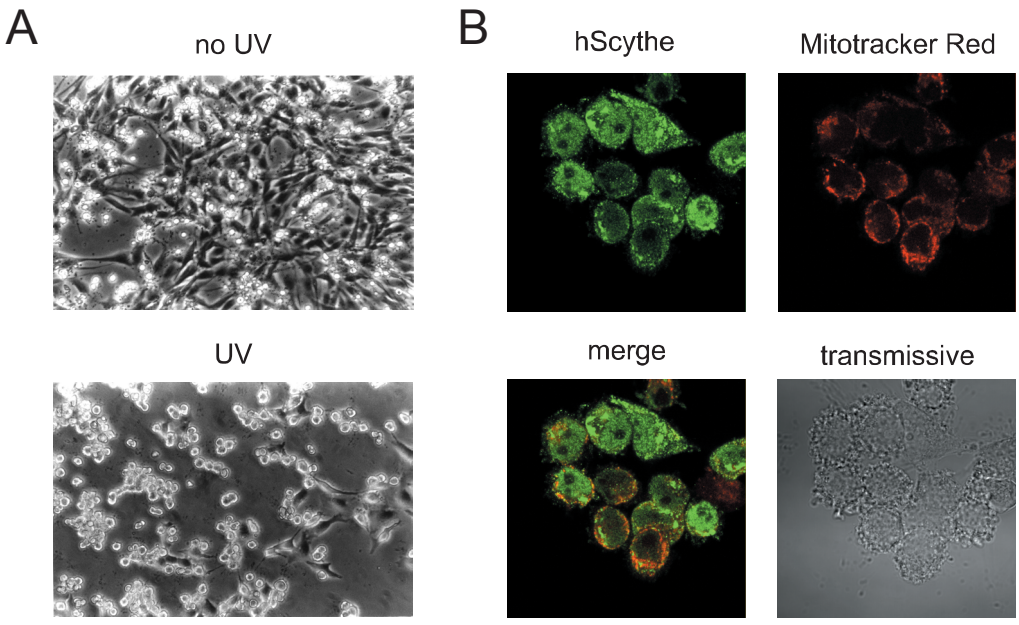


**Figure 5. Effect of MG132 and cycloheximide on the localization of endogenous hScythe.**
**(A)** Cells were incubated for 3 hours with 20 mM MG132 or 350 mM cycloheximide. 30 min before fixation MitoTracker Red was added to the medium, cells were fixed, permeabilized, and stained with anti-hScythe antibody. Detection was facilitated using an Alexa 488 conjugated secondary antibody and visualized using confocal laser scanning microscopy.
**(B)** Cells were incubated for the indicated time periods with 350 mM cycloheximide. Cells were lysed and total cell lysate was analyzed by western blotting with anti-hScythe antibody. Relative Molecular weight standards (Mr x 10-3) are shown at the left. WB, Western blot detection.

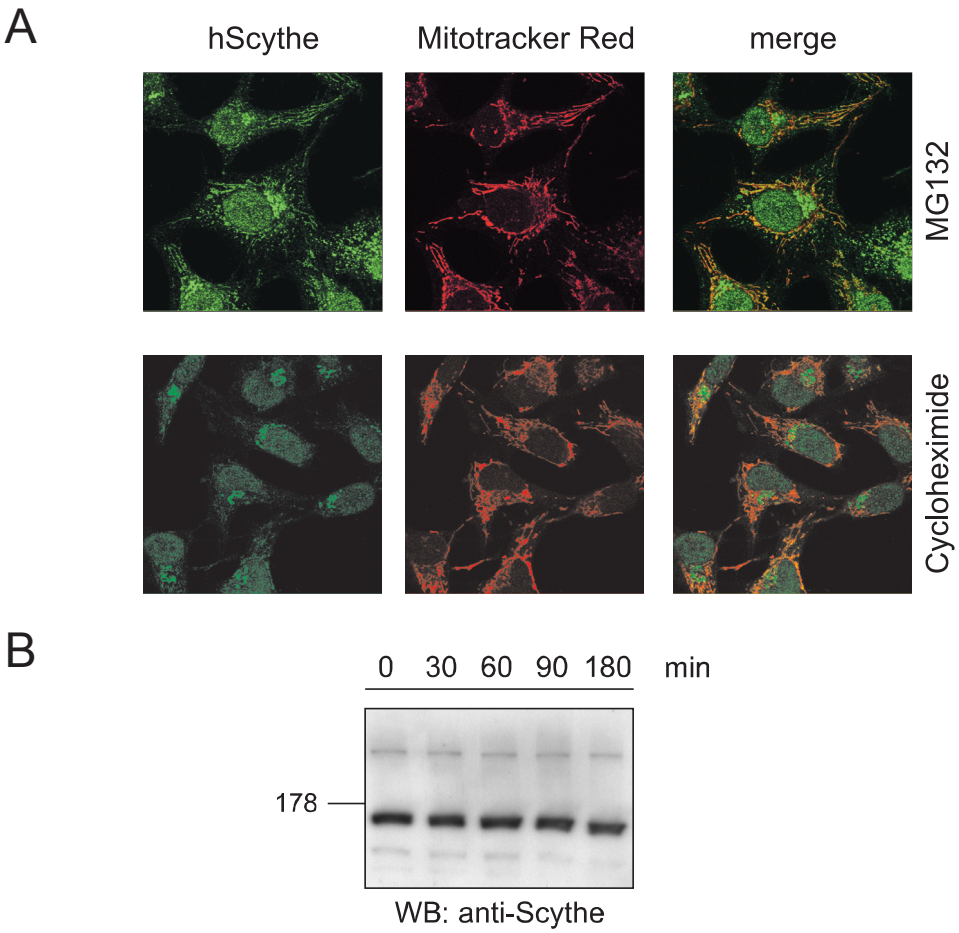



**Figure 4. Subcellular localization of endogenous hScythe after induction of apoptosis.**
**(A)** Cells were serum starved for 20 hours before UV radiation, and treated with 400J/m2 UVC. Cells were incubated for 2 hours before addition of MitoTracker Red to the medium. After 2.5 hours cells were analyzed by phase contrast microscopy.
**(B)** Cells were fixed, permeabilized, and stained with anti-hScythe antibody.

In summary, we identified an anti-apoptotic regulator as a binding partner for the GHR, which might very well be involved in the anti-apoptotic action exerted by GHR. Furthermore, we localized hScythe to the nucleus, Golgi and to mitochondria, a central place in apoptosis regulation. Inhibition of protein synthesis results in a reduction of hScythe localization to mitochondria, in line with a function in the regulation of apoptosis.

## Acknowledgment

We would like to thank Sally Kornbluth for supplying the hScythe construct and Jord Stam for help with the apoptosis induction experiments. Jürgen Gent, Peter van Kerkhof, and Martin Sachse we thank for stimulating discussions. This work was supported by grants of the Netherlands Organization for Scientific Research (NWO-902-23-192), and a European Union Network grant (ERBFMRXCT96-0026).

## Abbreviations

GH, growth hormone; GHR, growth hormone receptor; GST, glutathione S-transferase; IAP, inhibitor of apoptosis; JAK, Janus kinase; NLS, nuclear localization signal; STAT, signal transducers and activators of transcription; TMD, transmembrane domain; UBD, ubiquitin-like domain; UbE, ubiquitin-dependent endocytosis;

## References

1. Casanueva, F. (1992) *Endocrinol. Metab. Clin. North Amer.* **21**, 483-517
2. Muller, E. E., Locatelli, V., and Cocchi, D. (1999) *Physiol. Rev.* **79**, 511-607
3. Waters, M. J., Shang, C. A., Behncken, S. N.*, et al.* (1999) *Clin. Exp. Pharmacol. Physiol.* **26**, 760-764.
4. Davidson, M. B. (1987) *Endocrinol. Rev.* **8**, 115-131
5. Baixeras, E., Jeay, S., Kelly, P. A.*, et al.* (2001) *Endocrinol.* **142**, 2968-2977.
6. Leung, D. W., Spencer, S. A., Cachianes, G.*, et al.* (1987) *Nature* **330**, 537-543
7. Kelly, P. A., Djiane, J., Postel-Vinay, M. C.*, et al.* (1991) *Endocrinol. Rev.* **12**, 235-251
8. Argetsinger, L. S., Campbell, G. S., Yang, X. N.*, et al.* (1993) *Cell* **74**, 237-244
9. Pawson, T., and Schlessinger, J. (1993) *Curr. Biol.* **3**, 434-442
10. Smit, L. S., Meyer, D. J., Billestrup, N.*, et al.* (1996) *Mol. Endocrinol.* **10**, 519-533
11. Campbell, G. S., Meyer, D. J., Raz, R.*, et al.* (1995) *J.Biol.Chem.* **270**, 3974-3979
12. Wood, W. I., Sliva, D., Lobie, P. E.*, et al.* (1995) *J. Biol. Chem.* **270**, 9448-9453
13. VanderKuur, J. A., Butch, E. R., Waters, S. B.*, et al.* (1997) *Endocrinol.* **138**, 4301-4307
14. Argetsinger, L. S., Norstedt, G., Billestrup, N.*, et al.* (1996) *J.Biol.Chem.* **271**, 29415-29421

15. Jeay, S., Sonenshein, G. E., Kelly, P. A., *et al.* (2001) *Endocrinol.* **142**, 147-156.
16. Jeay, S., Sonenshein, G. E., Postel-Vinay, M. C., *et al.* (2000) *Mol. Endocrinol.* **14**, 650-661.
17. Murphy, L. J., and Lazarus, L. (1984.) *Endocrinol.* **115**, 1625-1632.
18. Govers, R., van Kerkhof, P., Schwartz, A. L., *et al.* (1997) *Embo J.* **16**, 4851-4858
19. Sachse, M., van Kerkhof, P., Strous, G. J., *et al.* (2001) *J. Cell Sci.* **114**, 3943-3952.
20. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinol.* **143**, 1243-1252
21. Govers, R., ten Broeke, T., van Kerkhof, P., *et al.* (1999) *Embo J.* **18**, 28-36
22. van Kerkhof, P., Sachse, M., Klumperman, J., *et al.* (2001) *J. Biol. Chem.* **276**, 3778-3784
23. van Kerkhof, P., Alves dos Santos, C. M., Sachse, M., *et al.* (2001) *Mol. Biol. Cell* **12**, 2556-2566
24. Banerji, J., Sands, J., Strominger, J. L., *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2374-2378
25. Manchen, S. T., and Hubberstey, A. V. (2001) *Biochem. Biophys. Res. Commun.* **287**, 1075-1082
26. Ozaki, T., Hanaoka, E., Naka, M., *et al.* (1999) *DNA Cell Biol.* **18**, 503-512
27. Thress, K., Song, J., Morimoto, R. I., *et al.* (2001) *Embo J.* **20**, 1033-1041
28. Takayama, S., and Reed, J. C. (2001) *Nature Cell Biol.* **3**, E237-241
29. Takayama, S., Xie, Z., and Reed, J. C. (1999) *J. Biol. Chem.* **274**, 781-786
30. Kaye, F. J., Modi, S., Ivanovska, I., *et al.* (2000) *FEBS Lett.* **467**, 348-355
31. Thress, K., Henzel, W., Shillinglaw, W., *et al.* (1998) *Embo J.* **17**, 6135-6143
32. Thress, K., Evans, E. K., and Kornbluth, S. (1999) *Embo J.* **18**, 5486-5493
33. McCarthy, J. V., and Dixit, V. M. (1998) *J. Biol. Chem.* **273**, 24009-24015
34. Strous, G. J., van Kerkhof, P., Govers, R., *et al.* (1996) *Embo J.* **15**, 3806-3812
35. van Kerkhof, P., Govers, R., Alves dos Santos, C. M., *et al.* (2000) *J. Biol. Chem.* **275**, 1575-1580
36. Shevchenko, A., Wilm, M., Vorm, O., *et al.* (1996) *Anal. Chem.* **68**, 850-858
37. Meiring, H. D., van der Heeft, E., ten Hove, G. J., *et al.* (2002) *J. Sep. Sci.* **25**, 557-568
38. Stam, J. C., Geerts, W. J., Versteeg, H. H., *et al.* (2001) *J. Biol. Chem.* **276**, 25176-25183
39. Kiess, W., and Gallaher, B. (1998) *Eur. J. Endocrinol.* **138**, 482-491
40. Jeay, S., Sonenshein, G. E., Postel-Vinay, M. C., *et al.* (2002) *Mol. Cell Endocrinol.* **188**, 1-7
41. Holley, C. L., Olson, M. R., Colon-Ramos, D. A., *et al.* (2002) *Nature Cell Biol.* **4**, 439-444
42. Somosy, Z. (2000) *Micron* **31**, 165-181

# Chapter IV

## The growth hormone receptor interacts with its sheddase, the tumor necrosis factor-α converting enzyme (TACE)

Julia A. Schantl*, Marcel Roza*, and Ger J. Strous*

*Department of Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands

*(Manuscript submitted)*

# Abstract

Proteolysis of the growth hormone receptor (GHR) occurs at the cell surface and results in the release of its extracellular domain, the growth hormone binding protein (GHBP). Tumor necrosis factor-α converting enzyme (TACE) has been identified as a putative protease responsible for GHR cleavage. However, the exact cleavage site and the way GHR and TACE interact are still unresolved. In this study, we identify TACE in Chinese hamster cells and show that TACE is correctly processed and transported to the cell surface. Interaction between GHR and TACE can only be observed when cells are pre-incubated with GH. As previously shown, GH binding to GHR induces a conformational change that inhibits the enzymatic activity of TACE. Our data show that the GHR-TACE interaction is still possible. Attempts to show an interaction between TACE and a protease insensitive GHR mutant, in which three amino acids of the extracellular domain were deleted, did not result in co-immunoprecipitation neither in the presence nor in the absence of GH. In summary, these results indicate that TACE binds to the extracellular domain of the GHR, presumably close to the transmembrane domain, and that the proteolytic protection induced by GH binding does not prevent substrate binding by TACE.

# Introduction

Postnatal growth as well as lipid and carbohydrate metabolism are regulated by growth hormone (GH), a peptide hormone synthesized primarily by the anterior pituitary gland [1]. GH exerts its action on target cells through the growth hormone receptor (GHR), which is ubiquitously expressed throughout the body with high levels in liver and adipose tissue [2]. The GHR, a 130 kDa type I plasma membrane protein, belongs to the cytokine receptor superfamily based on homologies in the extracellular domain and the lack of intrinsic kinase activity. Like many important hormones, the actions of GH are highly regulated at several levels. Secretion of GH is tightly controlled by two hypothalamic hormones, growth hormone releasing hormone (GHRH), which stimulates secretion, and somatostatin, which inhibits secretion [3]. GH hyposecretion during early postnatal development results in dwarfism, whereas hypersecretion before puberty leads to gigantism [4]. In addition to effects on growth, up-regulated GH secretion results in impaired cardiovascular functions, and is associated with metabolic disorders like glucose intolerance and diabetes mellitus as well as with certain types of cancer [5-7]. The effectiveness of a peptide hormone depends equally on its presence in the circulation as well as on the abundance of its receptor at the plasma membrane of its target cells. The latter depends on the one hand on the rate of synthesis of new receptor in the ER, receptor processing in the Golgi complex and transport to the plasma membrane and on the other hand on the rate of GHR removal from the cell surface. Two processes are known to contribute to receptor removal: (1) receptor internalization leads to degradation in the lysosome whereas (2) proteolysis at the cell surface results in the release of the extracellular domain of the GHR referred to as GH binding protein (GHBP) [8]. The latter process is also known as ectodomain shedding [9].

The physiological function of GHBP is unknown. Its abundance in the circulation probably reflects the GHR abundance in the system, with major contributions from liver [9]. GH can still bind to GHBP with high affinity. The GHBP-GH complex may serve as a circulating GH reservoir [10] that protects GH from degradation and excretion [11]. This mechanism can prolong the half-life of GH and may enhance its bioactivity *in-vivo* [12]. Moreover, GHBP may act as a modulator or inhibitor of GH action at the tissue level by competing with GHR for its ligand [13] or through formation of unproductive GHR-GHBP heterodimers that are unable to signal.

Depending on the species, formation of GHBP occurs through two distinct mechanisms. In rats or mice the GHBP is mainly secreted as an alternatively spliced

product of the GHR gene [14,15], however, rodent GHR is not completely resistant to proteolysis [16]. The alternatively spliced GHBP contains a short hydrophobic sequence in place of the transmembrane and intracellular domain. This hydrophobic tail is encoded by a special exon (exon 8A), which is missing in many other species including rabbits and humans [17,18]. Therefore, in rabbits and humans, GHBP results exclusively from proteolytic cleavage of the membrane anchored GHR [9]. In monkeys, and likely in rats and mice, both processes occur, but their relative contribution to the production of GHBP is unknown [19].

Shedding of the GHBP involves the action of the tumor necrosis factor-α converting enzyme (TACE), a transmembrane metalloprotease [20]. Ectodomain shedding of the GHR can be promoted by phorbol esters presumably through a pathway involving the protein kinase Cα and downstream activation of MAP kinases [21]. However, GH binding probably inhibits GHR shedding by inducing a conformational change of the GHR, and thereby rendering the receptor inaccessible for TACE [8,22]. Cleavage of the GHBP most likely takes place at the plasma membrane, because a truncated form of the GHR (GHR 1-279) with a prolonged residence time at the cell surface is particularly prone for shedding [23,24]. The exact mechanism by which TACE recognizes the GHR is still unknown. Sequence comparison of GHRs that are prone to shedding and those that are not yield no clue as to the cleavage site. Recently, a three amino acid juxta-membrane region in the extracellular domain of the GHR (E242-D244) has been reported to be a structural determinant required for shedding [25].

In this study, we show that TACE is present in Chinese hamster cells, and can be processed and transported to the plasma membrane. Furthermore, we have identified the mature, plasma membrane species of TACE that is able to interact with the GHR in the presence of GH *in vivo*.

# Material and Methods

### *Material*

Full-length rabbit GHR cDNA in pCB6 has been described [26]. Anti-GHR antibody was raised against amino acids 327–493 of the cytosolic tail of the GHR [26]; anti-$TACE_{cyto}$ antibody against the cytoplasmic domain of TACE was a generous gift of Carl Blobel (Memorial Soan-Kettering Cancer Center, New York). MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was purchased from Calbiochem-Novabiochem. Culture media, fetal calf serum (FCS), and antibiotics for tissue culture were purchased from Gibco.

**Table 1.** Nucleotide sequences of the primers used in this study

| Primers | Nucleotide sequence |
|---|---|
| A | 5′-CATTCACATGTGAAGTTCCGGTTTGCATGGTTCTTAATTATTATC |
| B | 5′-GATAATAATTAAGAACCATGCAAACCGGAACTTCACATGTGAATG |
| C | 5′-CCCATTCACATGTTTCCGGTTTCCATGGTTCTTAATTATTATC |
| D | 5′-GATAATAATTAAGAACCATGGAAACCGGAAACATGTGAATGGG |

### *Construction of GHR(Δ E242–D244)*

To obtain the E242–D244 deletion construct of the GHR, we performed two rounds of Quick Change site-directed mutagenesis (Stratagene) PCR. In the first reaction we used primer A and B (Table 1), and in the second reaction we used primers C and D. The final construct was verified by *in-vitro* transcription translation.

### *Mammalian cells and transfection*

Chinese hamster ts20 cells were propagated at 30°C with MEMα supplemented with 4.5 g/l glucose, 10% fetal calf serum, 10 U/ml penicillin, and 100 µg/ml streptomycin. For transfection experiments, ts20 cells were grown in 60-mm dishes to 30–40% confluency before transfection with 5 µg DNA/dish, using Fugene (Roche) according to the manufacturer's protocol. 32 hours after transfection, cells were treated with 10 mM butyrate to enhance expression of CMV-driven constructs. Cells were used for experiments 48 hours after transfection.

### *Glycoprotein isolation*

Chinese hamster ts20 cells were lysed on ice in buffer A (0.5% Triton X-100, 50 mM NaF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM PMSF, 2 µM MG132). After clearing by centrifugation at 2000 rpm at 4°C, samples were precipitated using ConcanavalinA (ConA) beads (Amersham Pharmacia). Precipitated material was separated by SDS PAGE together with total cell lysate, transferred to polyvinylidene difluoride paper and immunostained with anti-$TACE_{cyto}$ antibody. After incubating the blots with protein-A conjugated to HRP, antigens were visualized using the ECL system (Boehringer Mannheim).

### *Cell surface biotinylation*

Chinese hamster ts20 cells were washed three times with ice-cold PBS, followed by a 30 min incubation on ice with 0.5 mg/ml Sulfo-NHS-SS-biotin (Pierce). The cells were washed twice with ice-cold PBS and lysed in buffer B (0.5% (w/v) Triton X-100, 50 mM NaF, 1mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM PMSF, and 2 µM MG132 in PBS). After clearing by centrifugation at 2000 rpm at 4°C, samples were precipitated using streptavidin beads (Pierce). Precipitated material was separated by SDS PAGE together with total cell lysate, transferred to polyvinylidene difluoride paper and immunostained with anti-$TACE_{cyto}$ antibody. After incubating the blots with protein-A conjugated to HRP, antigens were visualized using the ECL system (Boehringer Mannheim).

### *Co-immunoprecipitation*

Cells were lysed on ice in buffer A. The lysates were cleared by centrifugation 2000 rpm at 4°C. Reactions were incubated with GHR antiserum for 2 hours at 4°C. Protein-A agarose beads (Repligen Co) were used to isolate the immunocomplexes. The immunocomplexes were washed once with buffer A. Immunocomplexes and total cell lysates were analyzed on 7.5% SDS PAGE together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained with either anti-GHR or anti-$TACE_{cyto}$ antibody. After incubating the blots with protein-A conjugated to HRP, antigens were visualized using the ECL system (Boehringer Mannheim). To ascertain the significance of the results, the experiments were repeated 4–5 times. The figures show representative Western blot results.

## Results and Discussion

### *Characterization of TACE in Chinese hamster ts20 cells*

TACE is produced as an approximately 120 kDa proform, which is cleaved by a furine-like enzyme in the Golgi complex. It is this cleaved 100 kDa active form of TACE that is residing at the cell surface and is involved in the shedding of many substrates. To confirm the presence of TACE in Chinese hamster ts20 cells, immunoblot analysis of cellular extracts was performed. Using an antibody directed against the cytosolic domain of TACE, we detected two bands both in cell lysate and after immunoprecipitations with anti-TACE antibody: one at approximately 120 kDa and one at 100 kDa representing the proform and the processed active form of TACE (Fig. 1, lanes 2 and 4). To validate that the 120 kDa band is the glycosylated, uncleaved proform of TACE, precipitation of total cell lysate with Concanavalin-A-coated beads was performed. Concanavalin-A preferentially binds mannose and glucose residues with high affinity, mainly present in high-mannose containing proteins of ER and Golgi complex. Precipitation with Concanavalin-A beads resulted mostly in the isolation of an approximately 120 kDa band, which represents the uncleaved, ER glycosylated proform of TACE (Fig. 1, lane 5). Using cell biotinylation with membrane-impermeable activated biotin, we characterized the cell surface resident form of TACE. Only the active, furin-cleaved 100 kDa form of TACE was detected at the cell surface (Fig. 1, lane 3). The 100 kDa form of TACE at the cell surface represented only a small amount of total TACE in the cell. This is in agreement with studies on the localization of TACE indicating that the major pool of TACE resides in an intracellular perinuclear compartment, with some diffuse localization consistent with surface staining [27]. Doedens *et al.* described that only

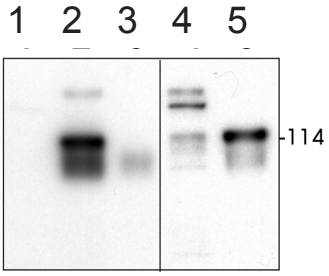



**Figure 1. Characterization of TACE in Chinese hamster ts20 cells.**
Cells, grown in 6 cm dishes, were lysed and the lysate was either directly detected with anti-TACE (lane 4) or after immunoprecipiation with the same antiserum (lane 2). In lane 3, the cells were biotinylated and TACE immunoblotting was performed after isolation with streptavidin beads. In lane 5, the cell extract was incubated with Concanavalin-A beads, and the bound TACE was analyzed by immunoblotting. In lane 1, immunoprecipitation was performed with pre-immune serum.

processed TACE appears at the cell surface [28]. In summary, these experiments confirm that TACE is present in Chinese hamster cells and that TACE is correctly processed and transported to the cell surface.

### *Co-immunoprecipitation of mature, cell surface resident TACE with GHR*

TACE was recently shown to be involved in the proteolytic cleavage of the GHR extracellular domain thereby generating the GHBP [20]. To investigate whether TACE is able to interact with the GHR directly, we performed co-immunoprecipitation experiments using ts20 cells transiently transfected with either empty vector or wt rabbit GHR cDNA. Co-immunoprecipitations were carried out with an antibody directed against epitopes 300 amino acids downstream of the plasma membrane in the cytosolic tail of the GHR or the corresponding pre-immune serum. TACE co-immunoprecipitation with the GHR antibody was only possible if GH was present (Fig, 2A, right panel). In the absence of GH no TACE was detectable. Only the cleaved, plasma membrane resident form of TACE interacted with GHR. Preimmune serum was not able to precipitate GHR nor could we detect nonspecific binding of TACE under these conditions. Cell lysates of cells transfected with rabbit GHR were detected with anti-GHR antibody demonstrating that the amount of expressed GHR is comparable in these cells (Fig. 2B, left panel). Thus, only the cell-surface located form of TACE recognizes the mature GHR.

GH effectively protects the GHR against proteolytic degradation both by TACE and other proteases like proteinase K and trypsin [8,22]. This protective effect is probably due to a conformational change brought about by the formation of a ternary complex of two GHR molecules and one GH molecule. Therefore, our findings indicate that TACE

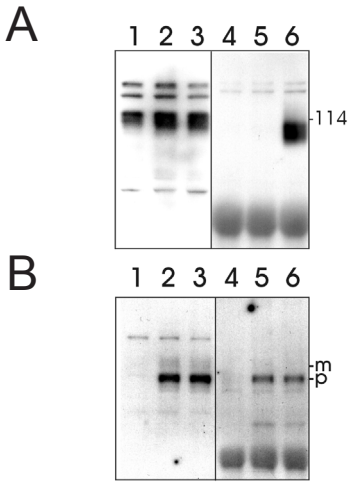



**Figure 2. Co-immunoprecipitation of TACE with anti-GHR antiserum.**
Cells, grown in 6 cm dishes, were transfected with either empty vector (panels A and B, lanes 1 and 4) or with wtGHR cDNA (all other lanes). In A and B, lanes 3 and 6, cells were incubated for 10 min with GH. After lysis, small aliquots of the cell extracts were applied to SDS-PAGE (panel A, lanes 1-3), and the remaining extracts were immunoprecipitated with anti-GHR and analyzed by immunoblotting with anti-TACE (panel A, lanes 4-6). In panel B, the blot of (A) was stripped and reprobed for GHR: in lanes 2-3, and 5-6, equal amounts of GHR species were expressed (p, indicating the precursor GHR (110 kDa), and m, mature GHR (130 kDa)). Relative molecular weight standard (Mr x 103) is indicated at the right.

still recognizes the GHR when GH is bound to the receptor. For that reason, the inhibitory effect of GH binding on shedding is not caused by an inhibition of TACE interaction with the receptor but rather an inhibition of enzyme function. Possibly, the conformational change in the receptor molecules results in the protection of the cleavage site in the GHR, which was proposed to be distinct from the site of interaction [29]. Furthermore, it can also be concluded that the interaction between the GHR and TACE is very transient under conditions when cleaveage occurres, because our experiments show that the GHR-TACE interaction was only detectable if the cleavage was inhibited by GH. This explains why we were only able to find an interaction between TACE and the GHR after binding of GH.

Recently, three residues (E242-D244) located close to the transmembrane domain of the GHR have been identified as a structural prerequisit for cleavage [25]. These three amino acids of the GHR do not represent the cleavage site for TACE, because mutation of these charged residues into alanines does not alter the release of GHBP. However, deletion of these three residues abolishes GHBP shedding probably by limiting the access of TACE to the cleavage site. Despite several attempts, we were not able to detect TACE binding to the GHR, in which the three juxta-membrane amino acids (E242-D244) were deleted (results not shown). These results are in agreement with the findings of Conte *et al.* suggesting that the juxta-membrane portion of the GHR constitutes or is part of the GHR-TACE interaction domain.

In summary, we show that mature, plasma membrane resident TACE can interact

with the GHR, when GH is bound. The conformational change of the GHR molecules after GH binding results in an inaccessible cleavage site, thereby protecting the GHR molecules from shedding. Deletion of amino acids 242-244 in the extracellular domain of GHR probably restricts the contact of TACE with the interaction site, which might explain the protection from proteolytic cleavage in this mutant GHR.

## Acknowledgments

We would like to thank Carl Blobel for supplying the TACE-antibody and Jürgen Gent, Peter van Kerkhof, and Martin Sachse for stimulating discussions. This work was supported by grants of the Netherlands Organization for Scientific Research (NWO-902-23-192), and a European Union Network grant (ERBFMRXCT96-0026).

## Abbreviations

ER, endoplasmic reticulum; GH, growth hormone; GHBP, growth hormone binding protein; GHR, growth hormone receptor; TACE, TNF-α converting enzyme; TNF, tumor necrosis factor.

## References

1. Casanueva, F. F. (1992) *Endcrinol. Metab. Clin. North Am.* **21**, 483-517
2. Kelly, P. A., Djiane, J., Postel-Vinay, M. C., *et al.* (1991) *Endocrinol. Rev.* **12**, 235-251
3. Muller, E. E., Locatelli, V., and Cocchi, D. (1999) *Physiol. Rev.* **79**, 511-607
4. Cheek, D. B., and Hill, D. E. (1974) (Knobil, E., and Sawyer, W. H., eds) Vol. 4, pt 2, pp. 159-185, American Physiology Society
5. Okada, S., and Kopchick, J. J. (2001) *Trends Mol. Med.* **7**, 126-132.
6. Domenech-Santasusana, M., Carles, J., Goday, A., *et al.* (1994) *Ann. Oncol.* **5**, 659
7. Beyan, C., Ural, A. U., Cetin, T., *et al.* (1996) *Endocrinol. J.* **43**, 565-568
8. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinol.* **143**, 1243-1252
9. Baumann, G. (2001) *J. Pediatr. Endocrinol. Metab.* **14**, 355-375.
10. Baumann, G., Amburn, K., and Shaw, M. A. (1988) *Endocrinol.* **122**, 976-984
11. Baumann, G., Amburn, K. D., and Buchanan, T. A. (1987) *J. Clin. Endocrinol. Metab.* **64**, 657-660
12. Clark, R. G., Mortensen, D. L., Carlsson, L. M., *et al.* (1996) *Endocrinol.* **137**, 4308-4315
13. Mannor, D. A., Winer, L. M., Shaw, M. A., *et al.* (1991) *J. Clin. Endocrinol. Metab.* **73**, 30-34
14. Baumbach, W. R., Horner, D. L., and Logan, J. S. (1989) *Genes Dev.* **3**, 1199-1205
15. Smith, W. C., Kuniyoshi, J., and Talamantes, F. (1989) *Mol. Endocrino.l* **3**, 984-990
16. Guan, R., Zhang, Y., Jiang, J., *et al.* (2001) *Endocrinol.* **142**, 1137-1147.
17. Zhou, Y., He, L., and Kopchick, J. J. (1994) *Receptor* **4**, 223-227
18. Edens, A., Southard, J. N., and Talamantes, F. (1994) *Endocrinol.* **135**, 2802-2805

19. Martini, J. F., Pezet, A., Guezennec, C. Y., *et al.*. (1997) *J. Biol. Chem.* **272**, 18951-18958
20. Zhang, Y., Jiang, J., Black, R. A., *et al.* (2000) *Endocrinol.* **141**, 4342-4348
21. Alele, J., Jiang, J., Goldsmith, J. F., *et al*. (1998) *Endocrinol.* **139**, 1927-1935
22. Zhang, Y., Guan, R., Jiang, J., *et al.* (2001) *J. Biol. Chem.* **17**, 17
23. Dastot, F., Sobrier, M. L., Duquesnoy, P., *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10723-10728
24. Ross, R. J., Esposito, N., Shen, X. Y., *et al.* (1997) *Mol. Endocrinol.* **11**, 265-273
25. Conte, F., Salles, J. P., Raynal, P., *et al.* (2002) *Biochem. Biophys. Res. Commun.* **290**, 851-857
26. Strous, G. J., van Kerkhof, P., Govers, R., *et al.* (1996) *Embo J.* **15**, 3806-3812
27. Schlöndorff, J., Becherer, J. D., and Blobel, C. P. (2000) *Biochem. J.* **347 Pt 1**, 131-138
28. Doedens, J. R., and Black, R. A. (2000) *J. Biol. Chem.* **275**, 14598-14607
29. Maskos, K., Fernandez-Catalan, C., Huber, R., *et al.* (1998) *Proc. Natl. Acad. Sci. U S A* **95**, 3408-3412

# Chapter V

## Summary

Julia A. Schantl

Department of Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands

# Abstract

Growth hormone (GH) is one of the central players in growth [1]. GH action depends not only on the presence of the hormone in the circulation but also on the availability of its receptor (GHR) at the cell surface of the target cells [2]. The maintenance of GH binding capacity depends on one hand on the synthesis of new receptor in the ER, receptor processing in the Golgi, and transport to the plasma membrane and on the other hand on the removal of GHR from the cell surface. Two processes are known to contribute to receptor turnover: (1) proteolysis at the cell surface results in the release of the GH binding protein (GHBP), whereas (2) receptor endocytosis leads to the removal of the receptor molecule from the cell surface followed by degradation in the lysosome. Shedding of the GHBP involves the action of the tumor necrosis factor-α converting enzyme (TACE), a transmembrane metalloprotease [3]. Endocytosis of the GHR is regulated by the ubiquitin-proteasome system and mediated via a 10 amino acid motif within the GHR cytosolic tail (UbE motif; DSWVEFIELD) [4]. Mutation of phenylalanine at position 327 into an alanine abolishes ubiquitination as well as internalization. The UbE motif can thus be considered a specific target for the ubiquitin system. Effectors of the GHR have been the subjects of investigation in this thesis. In particular, we have studied proteins that target the UbE motif of the GHR and the interaction of the GHR with its sheddase, TACE.

# Identification of new effectors of the GHR

Until recently, only proteins involved in signal transduction were known to bind directly (JAK2 and STAT5) or indirectly (STAT1 and STAT3) to the GHR. The abundance of GHRs is not controlled by these proteins, because Box1 mutants defective in JAK2 binding have a similar half-life and trafficking characteristics. GHR Box1 mutants are controlled by the ubiquitin system in a similar way as the wt GHR [5].

In order to identify new proteins that are involved in the ubiquitination and internalization of the GHR and interact with the UbE motif in the GHR we performed *in-vitro* pull-down experiments. Using GST-fusion proteins encompassing the UbE motif, we have isolated two new downstream effectors of the GHR, which were identified by mass spectrometry: small glutamine rich tetratricopeptide repeat containing protein (SGT) and Scythe.

### *Small glutamine-rich tetratricopeptide repeat-containing protein*

SGT was originally discovered as a protein interacting with envelope proteins of two viruses, namely parvorvirus H-1 and human immunodeficiency virus type 1 [6,7]. Sequence analysis of SGT revealed three-tandem tetratricopeptide repeats (TPR). TPR motifs are highly degenerated 34 amino acid repeats involved in protein-protein interaction [8]. We have shown that SGT binds specifically to the UbE motif of the GH, both *in-vitro* and *in-vivo* (Chapter II). We isolated four SGT species with different pI. Because SGT contains three TPR motifs, these modifications might occur in the TPR motif themselves. The interaction between the UbE motif of the GHR and SGT takes place through the first TPR motif of SGT. Deletion of TPR1 resulted in a loss of interaction with the UbE motif. The remaining TPR motifs were unable to substitute for TPR1 indicating that these TPR motifs are involved in interactions with other proteins. An alternative explanation for the loss of interaction is that the deletion of the first TPR motif results in the disturbance of the overall structure of the TPR motifs thereby preventing the interaction. Furthermore, we have identified a pentatricopeptide repeat (PPR) containing protein, LRP130, which can bind specifically to the UbE motif. PPR motifs show high similarity with TPR motifs [9], indicating that the UbE motif can specifically interact with proteins that contain this kind of protein-protein interacting motif.

*In-vivo,* SGT can interact both with the precursor and the mature form of the GHR. This indicates that the interaction can already occur in the ER, possibly as early as during

translation and dimerization of the GHR. It is rather unlikely that SGT is involved in protein folding, because a negative effect on the folding activity of Hsc/Hsp70 has been established for SGT. Furthermore, the GHR mutant F327A does not bind SGT, but shows no defect in receptor dimerization, indicating that SGT has no role in receptor dimerization. So far, we cannot exclude that SGT plays a role in the transport of the GHR to the plasma membrane as well as in the internalization and sorting to the lysosomes, where the GHR is degraded. However, we have no data to support these hypotheses.

The ubiquitin system, which is involved in the internalization of the GHR, does not affect the interaction between SGT and the GHR, meaning that the GHR-SGT interaction might precede the action of the ubiquitin system. It is possible that SGT interacts with the UbE motif on GHR's way from the ER to the cell surface. Thereby, SGT prevents the interaction of the ubiquitin system with the GHR, until a trigger releases SGT from the receptor and the ubiquitin system can come into action.

The TPR motifs within SGT might give an indication to its function. TPR containing proteins have been linked with the ubiquitin system. C-terminus of Hsc/Hsp70 interacting protein (CHIP) shows E3 ubiquitin ligase activity [10], or sgt1 is able to interact with subunits of the SCF E3 ligase complex [11]. Therefore, it is tempting to speculate that SGT interacts with components of the ubiquitin-proteasome system and link the GHR to the ubiquitin-proteasome system, thereby controlling the downregulation of the GHR by competing for the UbE motif.

### *Scythe*

Scythe, previously termed Bat3, was originally identified as one of the genes located in the human major histocompatibility complex [12]. The N-terminus of Scythe encodes a ubiquitin-like domain, and at the C-terminus a nuclear localization signal and BAG domain, which is involved in interactions with Hsc70, were identified [13,14]. Binding of Scythe to Hsc/Hsp70 inhibits the protein folding activity of Hsc70. Reaper, a potent apoptotic regulator, can reverse the inhibitory effect of Scythe on Hsc/Hsp70 [14]. Scythe also functions in the regulation of apoptosis, probably by sequestering a pro-apoptotic factor. Binding of Reaper to Scythe results in the release of this factor, which then triggers cytochrome c release from mitochondria. We isolated Scythe as a protein that specifically binds to the UbE motif of the GHR *in-vitro* (Chapter III). Due to high unspecific binding of Scythe to IgG we were unable to reproduce these data *in-vivo*, nor could we identify the domain that is responsible for this interaction. Nevertheless, Scythe might be an important downstream factor of the GHR involved in the anti-apoptotic

action of GH, exerted by the GHR [15].

Furthermore, we studied the localization of endogenous Scythe in HeLa cells. Beside the localization of Scythe in the nucleus due to its nuclear localization signal, we observed Scythe in the Golgi area and on mitochondria. Mitochondria play an important role in the induction of apoptosis via disruption of cellular energy metabolism and/or release of pro-apoptotic agents, like cytochrome c, Smac/DIABLO, endonuclease G, and Omi/HtrA2. Members of the Bcl-2 protein family control the integrity and response of mitochondria to apoptotic signals. The Bcl-2 associated anthogene-1 (BAG-1) shares several features with Scythe, like the N-terminal ubiquitin-like domain and the C-terminal BAG domain. Immunofluorescence confocal microscopy studies indicated that BAG-1 localizes to either cytosol, nucleus or both, depending on the particular cell type. In some cases, cytosolic BAG-1 immuno-staining was clearly associated with organelles resembling mitochondria, which is consistent with the finding that BAG-1 interacts with Bcl-2 and related proteins [16].

### *SGT and Scythe acting together?*

SGT and Scythe share one common feature: they are both co-chaperones of Hsc70, which negatively affect the folding activity of this chaperone when bound to Hsc70. Two other co-chaperones of Hsc70, CHIP (C-terminus of Hsc/Hsp70 interacting protein) and BAG-1 (Bcl-2-associated anthogene-1), show the same hallmarks and are together involved in the ubiquitination and degradation of chaperone substrates, such as cystic fibrosis transmembrane conductance regulator (CFTR) and the glucocorticoid receptor [17,18]. CHIP is, like SGT, a TPR containing protein. Through this TPR domain CHIP is able to interact with Hsp70. Furthermore, it possesses a C-terminal U-box and a RING-finger like domain, which is responsible for CHIP's E3 ligase activity. CHIP mediates the removal of chaperone-selected proteins from the productive folding pathway and targets them for degradation by the proteasome via BAG-1. BAG-1 acts as a coupling factor between the molecular chaperones and the proteasome complex [19]. The ubiquitin-like domain appears to serve as a proteasomal targeting signal and enables BAG-1 to recruit Hsc/Hsp70 to the proteasome.

SGT and Scythe might work in a similar way as proposed for CHIP and BAG-1, except that SGT misses a U-box. This does not preclude that SGT might function as a linker to the enzymes of the ubiquitin machinery. Scythe could interact with the proteasome through its ubiquitin-like domain, although no evidence is available yet that Scythe is indeed able to interact with the proteasome.

How could this fit into a model for GHR function? Both the proteasome and the ubiquitin system are necessary for proper endocytosis of the GHR. At this moment we only know that SGT can directly interact with the GHR. SGT binds to the GHR through TPR1, and binds Hsc/Hsp70 through the remaining TPR motifs. Hsc/Hsp70 thereby acts as a linker to Scythe, which in turn attracts the proteasome. However, the UbE motif does not require the proteasome for the GHR endocytosis process. The sequence involved in the proteasome action is located between amino acids 369 and 399. Although this region might stabilize SGT binding, it is not required for *in-vitro* binding. Therefore, we must conclude that it is too early to fit these proteins into a model for GHR function. The identification of the enzymes involved in GHR ubiquitination might shed more light into the interplay of the ubiquitin-proteasome system, SGT and Scythe during ubiquitin-dependent endocytosis of the GHR.

## Tumor necrosis factor-α converting enzyme

TACE has recently been identified as an important enzyme for cleavage of the GHR and shedding of the GHBP [3]. TACE is produced as an inactive proform, which is cleaved by a furin-like enzyme in the Golgi. It is present at the plasma membrane where the cleaved active form of TACE is involved in shedding of many substrates [20]. We investigated the ability of the GHR to bind to TACE (Chapter IV). Co-immunoprecipitation of TACE with an antibody directed against the GHR was only possible after treatment of the cells with GH. GHR interacts exclusively with the plasma membrane resident, active form of TACE, indicating that the interaction takes place at the cell surface. GH effectively protects the GHR against proteolytic degradation both by TACE and other proteases like proteinase K and trypsin [2,21]. This protective effect is probably due to a conformational change brought about through the formation of a ternary complex of two GHR molecules and one GH molecule. Because binding of GH to its receptor is a prerequisite to observe the interaction with TACE, we conclude that the negative effect of GH binding on shedding results from the inhibition of TACE function. Possibly, the conformational change in the receptor molecules leads to the protection of the cleavage site in the GHR, which was proposed to be distinct from the site of interaction [22]. In addition, the interaction between TACE and the GHR in the absence of GH seems to be very transient, because we could only observe the interaction when cleavage was inhibited.

Shedding of GHBP by TACE can be stimulated by treatment with phorbol 12-

myristate 13-acetate (PMA), which stimulates the protein kinase C (PKC) pathway [23]. Furthermore, PKC inhibitors completely block GHBP shedding [24]. These observations indicate that PKC pathways play a critical role in TACE activation. One possibility is that PMA increases TACE exocytosis from its intracellular storage location. However, the mechanistic link between PKC and TACE activation is still not understood, especially because deletion of the cytoplasmic tail of TACE does not influence its shedding activity in response to PMA [25]. Interestingly, GH activates PKC through the GHR via the phospholipase C signal transduction pathway [26]. Thereby, GH might signal shedding of excess GHR at the cell surface. This could result in desensitizing of GHR for GH that is still in the circulation, thus terminating signal transduction. One could argue that in this way GH is involved in a feedback loop that negatively influences the availability of the GHR for signaling at the cell surface.

# References


1. Argetsinger, L. S., and Carter-Su, C. (1996) *Physiol. Rev.* **76**, 1089-1107
2. van Kerkhof, P., Smeets, M., and Strous, G. J. (2002) *Endocrinol.* **143**, 1243-1252
3. Zhang, Y., Jiang, J., Black, R. A.*, et al.* (2000) *Endocrinol.* **141**, 4342-4348
4. Govers, R., ten Broeke, T., van Kerkhof, P.*, et al.* (1999) *Embo J.* **18**, 28-36
5. Alves dos Santos, C. M., ten Broeke, T., and Strous, G. J. (2001) *J. Biol. Chem.* **276**, 32635-32641
6. Cziepluch, C., Kordes, E., Poirey, R.*, et al.* (1998) *J. Virol.* **72**, 4149-4156
7. Callahan, M. A., Handley, M. A., Lee, Y. H.*, et al.* (1998) *J. Virol.* **72**, 8461
8. Blatch, G. L., and Lassle, M. (1999) *Bioessays* **21**, 932-939
9. Small, I. D., and Peeters, N. (2000) *Trends Biochem. Sci.* **25**, 46-47
10. Jiang, J., Ballinger, C. A., Wu, Y.*, et al.* (2001) *J. Biol. Chem.* **276**, 42938-42944
11. Kitagawa, K., Skowyra, D., Elledge, S. J.*, et al.* (1999) *Mol. Cell* **4**, 21-33
12. Banerji, J., Sands, J., Strominger, J. L.*, et al.* (1990) *Proc. Natl. Acad. Sci. U S A* **87**, 2374-2378
13. Manchen, S. T., and Hubberstey, A. V. (2001) *Biochem. Biophys. Res. Commun.* **287**, 1075-1082
14. Thress, K., Song, J., Morimoto, R. I.*, et al.* (2001) *Embo J.* **20**, 1033-1041
15. Jeay, S., Sonenshein, G. E., Postel-Vinay, M. C.*, et al.* (2002) *Mol. Cell. Endocrinol.* **188**, 1-7
16. Takayama, S., Krajewski, S., Krajewska, M.*, et al.* (1998) *Cancer Res.* **58**, 3116-3131
17. Connell, P., Ballinger, C. A., Jiang, J.*, et al.* (2001) *Nature Cell. Biol.* **3**, 93-96
18. Meacham, G. C., Patterson, C., Zhang, W.*, et al.* (2001) *Nature Cell. Biol.* **3**, 100-105
19. Luders, J., Demand, J., and Hohfeld, J. (2000) *J. Biol. Chem.* **275**, 4613-4617
20. Baumann, G., and Frank, S. J. (2002) *J Endocrinol* **174**, 361-368
21. Zhang, Y., Guan, R., Jiang, J.*, et al.* (2001) *J. Biol. Chem.* **276**, 24565-24573
22. Maskos, K., Fernandez-Catalan, C., Huber, R.*, et al.* (1998) *Proc. Natl. Acad. Sci. U S A* **95**, 3408-3412
23. Guan, R., Zhang, Y., Jiang, J.*, et al.* (2001) *Endocrinol.* **142**, 1137-1147

24. Saito, Y., Takagi, K., Teshima, R.*, et al.* (1998) *Mol. Cell Endocrinol.* **146**, 197-205
25. Reddy, P., Slack, J. L., Davis, R.*, et al.* (2000) *J. Biol. Chem.* **275**, 14608-14614
26. Moutoussamy, S., Renaudie, F., Lago, F.*, et al.* (1998) *J. Biol. Chem.* 273, 15906-15912

# Nederlandse samenvatting

## -voor de leek-

Julia A. Schantl

Groei van het menselijk lichaam is een complex proces dat nauwkeurige controle vereist. Groeihormoon (GH) speelt hierin een belangrijke rol. Het is betrokken bij lengtegroei van kinderen en gedurende het hele leven bij het herstel van beschadigd weefsel, voor de opbouw en instandhouding van de spiermassa en voor de verbranding van overtollig vet. Een tekort aan GH (GH-deficiëntie) resulteert in groeivertraging (dwerggroei) en onder andere in een overmaat aan vetweefsel, met name rond de buik. Overproduktie van GH, een klinisch syndroom dat agromegalie wordt genoemd, leidt onder andere tot vergrote handen en voeten. GH wordt gemaakt in de hyperfyse-voorkwab en via de bloedbaan door de gehele lichaam getransporteerd. Het meeste GH wordt aangemaakt bij pasgeborenen. Na het twaalfde levensjaar gaat de hoeveelheid GH in de bloedcirulatie omlaag. Een verminderde GH-produktie wordt gezien als een belangrijke factor bij veroudering en achteruitgang van veel lichamelijke functies op hogere leeftijd.

Voor de signaalverwerking van GH in cellen moet de aanwezigheid van het hormoon in het bloed worden opgemerkt. Op het oppervlak van een cel (de celwand of de plasmamembraan) bevinden zich sensoren, zogenaamde receptoren, die reageren op de hoeveelheid van een bepaald hormoon in het bloed. Deze receptoren hebben een extracellulair deel, dat buiten de cel uitsteekt, een transmembraandomein, dat zorgt voor de verankering in de plasmamembraan, en een intracellulair deel, dat in de cel hangt. De groeihormoonreceptor (GHR) op het celoppervlak van bijvoorbeeld lever-, hart-, spier- of botcellen bindt GH dat in het bloed aanwezig is en geeft vervolgens een signaal door naar de celkern. Hier wordt het signaal vertaald in de aanmaak van eiwitten. Het initiëren van groei is niet alleen afhankelijk van de hoeveelheid hormoon in het bloed maar ook van de aanwezigheid van voldoende receptoren aan de plasmamembraan. Een cel kan de hoeveeldheid van een eiwit beïnvloeden door de aanmaak (synthese) en afbraak (degradatie) van een eiwit te reguleren. De balans tussen synthese en degradatie bepaalt uiteindelijk de absolute hoeveelheid van ieder individueel eiwit (GHR is net als GH een eiwit). De aanmaak van GHR en de snelheid van het transport door de cel naar de plasmamembraan bepalen de aanvoer van de GHR, terwijl twee processen betrokken zijn bij het verwijderen van de GHR van het celoppervlak respectievelijk het inaktief maken van de receptor die aan de plasmamembraan aanwezig is. Het verwijderen van de receptor van de plasmamembraan wordt door het ubiquitine-proteasoomsysteem geregeld terwijl het enzym TACE de buitenkant van de receptor verwijdert, waardoor GH niet meer aan de receptor kan binden.

Het ubiquitine-proteasoomsysteem is een van de belangrijkste eiwitafbraak-

systemen in de cel. Eiwitten, die uit de cel verwijderd moeten worden, omdat zij schadelijk of overbodig zijn voor de cel, worden door enzymen van het ubiquitine-systeem herkend. Deze enzymen markeren het af te breken eiwit met een ander klein eiwit, ubiquitine. Aan dit ubiquitine kunnen vervolgens meerdere ubiquitine-eiwitten gekoppeld worden en deze ubiquitineketen wordt nu door een groot eiwitafbraakcomplex, het proteasoom, herkend. Het proteasoom is in staat om dit eiwit in kleine stukken, peptides, af te breken. Deze worden door de cel hergebruikt voor het maken van nieuwe eiwitten. Recent onderzoek heeft aangetoond dat het ubiquitinesysteem eveneens de verblijftijd van sommige receptoren aan het celopervlak reguleert op een dusdanige wijze dat cellen niet teveel en niet te weinig signalen krijgen. Dit geldt ook voor de GHR. De receptor wordt regelmatig van het celoppervlak verwijderd zodat niet te veel receptoren aan zich het celoppervlak verzamelen. Daarvoor worden ubiquitine-eiwitten aan de receptor gekoppeld (ubiquitinilering), die aan de cel signaleren, dat deze receptor van de plasmamembraan verwijderd moet worden. Vervolgens wordt de receptor naar het binnenste van de cel verplaatst, een proces dat internalisatie of endocytose genoemd wordt. In de cel wordt de receptor via het vroege endosoom en het late endosoom naar het lysosoom getransporteerd, waar uiteindelijk de afbraak plaatsvindt. Een speciaal motief in de aminozuurvolgorde van de GHR, het UbE-motief, is belangrijk voor de herkenning van de receptor door het ubiquitinesysteem en voor het endocytoseproces (aminozuren zijn eiwitbouwstenen). Verandering (mutatie) van aminozuren in dit motief verhindert dat de receptor door het ubiquitinesysteem wordt herkend, waardoor de receptoren zich aan de plasmamembraan ophopen.

Het doel van deze studie was het zoeken naar eiwitten die betrokken zijn bij de regulatie van de ubiquitinilering van de receptor en de endocytose, omdat deze tot nu toe nog niet bekend waren. Hiervoor werd een kunstmatig eiwit gemaakt dat gedeeltelijk uit het UbE-motief bestaat. Net als bij vissen gebruikten wij dit eiwit als lokaas waarmee wij in ‘de zee’ van celeiwitten ‘visten’ naar eiwitten die het UbE-motief herkennen en kunnen binden. Nadat wij deze eiwitten hadden geïsoleerd, konden die eiwitten verder worden geïdentificeerd. Van deze eiwitten hebben we twee eiwitten nader onderzocht. In hoofdstuk II beschrijven wij het eiwit SGT, dat in staat is aan de GHR te binden als het UbE-motief intakt is. Een klein motief, TPR-motief genoemd, van ongeveer 34 aminozuren van SGT is verantwoordelijk voor deze interaktie met de GHR. De interaktie is onafhankelijk van het funktioneren van het ubiquitinesysteem en wij zien geen invloed op de opname van de GHR met de door ons gebruikte technieken. We weten nog niet zeker welke rol SGT in de regulatie van de GHR speelt, maar de interaktie bestaat al kort

na de synthese van de receptor in het binnenste van de cel. Het is mogelijk, dat SGT de receptor voor het ubiquitinesysteem beschermt zodat het ubiquitinesysteem pas op het juiste moment zijn werk kan uitoefenen. In hoofdstuk III beschrijven wij een andere eiwit, hScythe, dat ook aan het UbE-motief van de GHR kan binden. hScythe is een eiwit dat betrokken is bij het tegenhouden van cel-zelfmoord (apoptose) net als de GH en GHR. Apoptose is een mechanisme van het lichaam om cellen te verwijderen, die niet goed funktioneren en een gevaar voor het lichaam opleveren. Gebeurt dit niet, dan kan dit bijvoorbeeld kanker tot gevolg hebben. De precieze lokatie van natuurlijk (endogeen) hScythe in de cel was tot nu toe niet bekend. We zijn erin geslaagd aan te tonen, dat hScythe zich in de cel behalve in de celkern ook rond de celkern en op mitochondriën (een organel in de cel, te vergelijken met organen in het lichaam) bevindt. Dat is een opmerkelijk resultaat omdat mitochondriën ook een belangrijke rol spelen bij het initiëren van apoptose. Als we de eiwitsynthese uitschakelen met behulp van een chemische stof, verdwijnt hScythe van de mitochondriën, maar blijft nog wel zichtbaar in en rond de kern. Remming van de eiwitafbraak heeft geen effect op de lokalisatie van hScythe.

In hoofdstuk IV hebben wij onderzocht of de GHR zich aan het enzym TACE kan binden. Het afknippen van het extracellulaire domein van de GHR door TACE (‘shedding’ genoemd) inaktiveert de receptor. GHR kan dan GH niet meer binden en zendt daarom ook geen signalen meer naar de celkern. Heeft de intakte GHR echter het GH gebonden dan is de receptor beschermd tegen TACE. TACE wordt in de cel geproduceerd als een inaktieve vorm (de pro-vorm) die tijdens het transport naar de plasmamembraan wordt geaktiveerd. Allereerst hebben wij onderzocht of in de door ons gebruikte cellen TACE aanwezig is, of TACE wordt geaktiveerd tijdens het transport naar de plasmamembraan en of het ook aan de plasmamembraan aanwezig is. Dit blijkt allemaal het geval te zijn. Vervolgens hebben wij naar omstandigheden gezocht waaronder TACE de GHR bindt. Alleen onder omstandigheden waarbij GH aan zijn receptor gebonden was, zagen wij een interaktie tussen de GHR en TACE. Dit betekent dat TACE de GHR nog steeds bindt, hoewel het enzym na het binden van het hormoon aan de receptor niet meer in staat is om het extracellulaire deel van de receptor af te knippen. Het is bekend dat het binden van GH aan de GHR een structuurverandering teweeg brengt. Vermoedelijk is de plaats waar TACE knipt hierdoor beschermd, en vindt er geen shedding plaats. Dat betekent verder dat TACE aan een ander domein dan de knipplaats bindt en dat het binden van GH aan zijn receptor de interaktie tussen de GHR en TACE niet bemoeilijkt.

# Curriculum vitae

### *Personal*

| | |
|---|---|
| Name | Julia Angelika Schantl |
| Date and Place of Birth | June 23rd 1972, Innsbruck, Austria |
| Nationality | Austrian |

### *Education*

| | |
|---|---|
| 1982–1990 | High School;Bundesoberstufenrealgymnasium, Austria |
| 1989 | High School; West High School, Wisconsin, USA |
| 1990 | Program in English as a Second Language; University of Wisconsin, Department of English, Wisconsin, USA |
| 1990–1997 | M.Sc.– Biotechnology; Universität für Bodenkultur Wien, Austria |

### *Research Experience*

| | |
|---|---|
| 1996–1997 | Project: '*A molecular characterization of a mixed population in a nitrifying wastewater treatment reator*' Delft University of Technology, Kluyver Laboratory of Biotechnology, Department of Microbiology and Enzymology, The Netherlands (Prof. Dr. J. G. Kuenen) Supervisor:Dr. Ir. M. Jetten and Dr. S. Logemann |
| 1997 | Project: *'Molecular genetic analysis of root morphogenesis'* Universität für Bodenkultur Wien, Zentrum für Angewandte Genetik, Austria Supervisor: Prof. Dr. DI. M. Hauser |
| 1998–2002 | Project: '*Growth Hormone Receptor Interacting Proteins'* University Medical Center Utrecht, Department of Cell Biology, The Netherlands Promotor: Prof. Dr. G.J.A.M. Strous |

# List of Publications

Logemann, S., Schantl, J.A., Bijvank, S., van Loosdrecht, M., Kuenen, J.G., Jetten M. 1998 Molecular microbial diversity in a nitrifying reactor system without sludge retention. FEMS Microbiology Ecology 27: 239-249

Strous, G.J. and Schantl J.A. 2001 Beta-arrestin and Mdm2, unsuspected partners in signaling from the cell surface. Sci STKE 110:PE41

Schantl, J.A., Roza, M., de Jong, A.P., Strous, G.J. 2002 Small glutamine-rich tetratricopeptide repeat-containing protein interacts with the ubiquitin-dependent endocytosis motif of the growth hormone receptor (Manuscript submitted)

Schantl, J.A., Roza, M., de Jong, A.P., Strous, G.J. 2002 The anti-apoptotic protein Scythe interacts with the ubiquitin-dependent endocytosis motif of the growth hormone receptor and localizes to mitochondria. (Manuscript in preparation)

Schantl, J.A., Roza, M., Strous, G.J. 2002 The growth hormone receptor interacts with its sheddase, the tumor necrosis factor-α converting enzyme (Manuscript submitted)

# Acknowledgements

Who would have thought that attending a theater play would be the first step to this thesis? But that is how it all started. And now after all these years of pipetting the results are summarized in this book. I still cannot believe it. I had a good time in the department of Cell Biology. Here I would like to thank everybody who has contributed in his/her own way in the realization of this PhD thesis and all the good times we have had in the lab, at work or during all the social activities. Some persons I would like to thank individually.

First of all I owe much to my promotor and supervisor: Ger, I am very grateful that you gave me the opportunity to work in your lab and perform my PhD thesis. Your enthusiasm and support helped me through some difficult times. I much appreciated the fact that your door was always open to discuss the latest results and give them a place in one of our many models.

Of course there is also the growth hormone receptor team. Jürgen, it was a great time working together with you. Your critical view on my results, presentations, and our discussions were a valuable contribution to my thesis. Sorry for your red ears. It was fantastic to be your 'paranimf', good to practice for my own big day as well. At the moment you are preparing for the next milestone in your life. I wish you and Moniek a fantastic day in February and all the luck for your future together. Peter, you were a big source of support. You had always time to solve technical problems and discuss results that led to new creative ideas. Good luck in St. Louis. Martin, thanks for all your efforts to make EM-pictures to support my biochemical results. Unfortunately, my antibodies rarely worked, but still you did not refuse to try again. Good luck with your own thesis defense in February and then in Paris!! Toine, Erica, and Roland, you too contributed to this thesis in our endless discussions. Thanks! Janine, I enjoyed working with you. Unfortunately, mdm2 did not go along with our ideas, but all this and some more struggles did not diminish your love for science. I hope that you have a successful time as AIO at hematology in front of you. All the best for you!! Monique, you were the latest addition to the GHR lab and just made the big step into the AIO world. Good luck with your work on the GHR!!

And then there are my 'paranimfen': Dagmar and Marcel. Your support during the past years was extremely valuable. Therefore, I am extremely glad that you agreed to be my 'paranimfen'. Dagmar, es ist jetzt wieder vier Jahre her, daß wir uns im Celbiolab kennengelernt haben. Daraus ist eine wunderbare Freundschaft entstanden: Osterbrunches, Expo in Hannover, und vieles mehr. Zu meiner großen Freude bist Du wieder nach Utrecht zurück gekommen, um für die nötige Abwechslung von der Arbeit zu sorgen (oder war da noch ein Grund?!). Vielen Dank für unsere Saunaabende, 'Der kleinen Grenzverkehr' (ich habe mich köstlich amüssiert), und Deine Kopier- und Stapelhilfe. Viel Erfolg mit Deinem 3-D Projekt. Marcel, dat ik het zonder je hulp nooit

gered had, hoef ik je niet te vertellen. Ik heb heel erg genoten van onze samenwerking. Je hebt altijd meegedacht hoe een experiment misschien net iets beter of completer uitgevoerd kon worden. Ik wist dat ik altijd op je kon rekenen en dat heeft me veel steun gegeven in de laatste maanden. Bedankt!! Verder moet ik nog vaak aan ons Oostenrijk weekend denken. Ik zal in de toekomst daarvoor zorgen dat Hollandse kost voor je klaar staat en zeker geen 'Knödel'. Veel succes met je 'vis-experimenten' en met het bouwen van je huis.

Giel, je was een heel fijne AIO-kamerbuurman. Bedankt voor de kopjes thee, de discussies en de computerhulp. Karin, bedankt voor de gezellige tijd, in koffiepauzes en ook in de activiteiten-commissie. Gelukkig heeft Jürgen nu ook een grote tafel, zodat we elkaar tijdens de spellenavonden nog regelmatig zullen spreken.

Marc en René, thank you for your help with posters and the layout of this book and the scanning of my cover. Always ready to help.

But life does not end after work and there are some persons outside the lab that I want to thank. Als erstes meinen Eltern für ihr Verständnis und ihre Unterstützung während der letzten Jahre. Mama, die Leutaschbesuche waren Dir immer zu kurz. Trotzdem vielen Dank für Deine Mühe, um es mir an nichts fehlen zu lassen, damit ich meine Energiereserven wieder auftanken konnte. Unser GHR-Wochenende in der Leutasch war sehr schön. Und die Filmvorstellungen nicht zu vergessen, Papa. Vielen Dank für Deine schnellen Korrekturen und Deine konstruktive Kritik. Barbara, ich werde unseren weihnachtlichen Labormarathon nicht so schnell vergessen. Die Resultate van dieser erfolgreichen Zusammenarbeit findest Du im 2. Kapitel. Danke!!!!!

Piet en Leny, bedankt voor jullie hulp bij alle verhuizingen en verbouwingen. Bedankt voor jullie begrip voor mijn tijdgebrek in de laatste maanden, en dat jullie dan even langs kwamen voor een kopje thee. Door jullie en ook door Hyo, Kyung en Hans, en Hyke en Michel voelde ik me hier snel thuis en welkom in de familie. Leo, erg leuk dat jij de omslag van mijn proefschrift wilde maken. Hij is echt geslaagd. Oma Huijg, bedankt voor je kaarsjes, ik denk dat ze hebben geholpen. Ik ben heel erg blij, dat wij u ons huisje konden laten zien.

Lieve Barry, je had het niet echt gemakkelijk met mij gehad die laatste maanden. Mijn welzijn kwam altijd op de eerste plaats. Wat deed ik zonder jou?! Al die koppen thee, die de lange avonden wat gemakkelijker maakten, urenlang mijn typwerk lezen, en alles op het werk laten staan, als mijn computer weer kuren kreeg. Woorden schieten me te kort. Nu samen op vakantie en verder door het leven maakt hopelijk veel goed.