The Ubiquitin-Proteasome Pathway and the Regulation of Growth Hormone Receptor Availability

Het ubiquitine-systeem en de regulatie van het aantal groeihormoonreceptoren aan het celoppervlak

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Abbreviations

APC	anaphase-promoting complex
CIS	cytokine-inducible SH2-domain containing protein
CSF	colony-stimulating factor
DUB	deubiquitinating enzyme
E6-AP	E6-associated protein
EGF	epidermal growth factor
ENaC	epithelial sodium channel
ERK	extracellular regulated kinase
GH	growth hormone
GHBP	GH-binding protein
GHIS	GH-insensitivity syndrome
GHR	GH-receptor
HECT	homologous to E6-AP C-terminus
HPV	human papillomavirus
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
IRS	insulin receptor substrate
JAK	Janus kinase
MAP	mitogen activated protein
NEDD4	neuronal precursor cell expressed developmentally downregulated
NEM	N-ethylmaleimide
PDGF	platelet derived growth factor
PI	phosphatidyl inositide
PKC	protein kinase C
PTB	phosphotyrosine binding
RING	really interesting new gene
SH2	Src homology-2
SIRP	signal-regulatory protein
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TACE	tumor necrosis factor-α-converting enzyme
TCR	T-cell receptor
TRAF	tumor necrosis factor-receptor associated factor
UEV	ubiquitin-conjugating enzyme variant
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau.

Chapter I

THE UBIQUITIN-PROTEASOME PATHWAY AND THE REGULATION OF GROWTH HORMONE RECEPTOR AVAILABILITY

INTRODUCTION

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Contents Chapter I

The Control of Growth

The control of growth is one of the major physiological functions of the endocrine system. Many hormones and growth factors are involved in the regulation of this complex process and it is understood that multiple factors, both endogenous and exogenous, influence growth. Growth-promoting proteins in the anterior pituitary were first demonstrated by Evans and Long in 1921, followed by the observation that hypophysectomy was associated with growth retardation, which could be reversed with pituitary implants [1, 2]. In 1944, bovine growth hormone was isolated, followed by pituitary human growth hormone (hGH) in 1956 [3, 4]. Administration of purified preparations of hGH to GH-deficient patients showed a marked increase in height and changes in body composition [5]. Purified bovine GH was found to be ineffective in patients with short stature or hypophyseal disorders, which led to the recognition of species specificity of GH in the primate [6]. When purified bovine growth hormone was used to study its metabolic actions, it was shown that GH indirectly stimulated [^{35}S] sulfate incorporation into cartilage via a serum factor, which was initially called "sulfation factor" but was later renamed somatomedin [7]. The concept that GH indirectly stimulates growth, via production of somatomedins or insulin-like growth factors (IGF) by the liver, is known as the somatomedin hypothesis of GH action [8]. Later, it was proposed that the somatomedins were paracrine factors when it was demostrated that the production of somatomedins, under GH control, occurred in many cell types in a wide variety of tissues [9]. On the basis of observations that GH interacted directly with several peripheral tissues, the somatomedin hypothesis was challenged and a dual-effector model of GH action was proposed [10]. Recent evidence suggests that both endocrine and local paracrine/autocrine GH-IGF systems play roles in normal postnatal growth [reviewed in: 11]. Several experiments in rats, comparing the systemic delivery of IGF-1 and GH, showed that IGF-1 and GH have independent and differential functions [12]. Effects of GH are mediated via the GH receptor (GHR) and expression of GHR is a prerequisite for the local action of GH. The GHR is present throughout the body with highest expression levels found in liver and adipose tissue. The control of growth depends equally on the presence of the peptide hormone in the circulation as well as the availability of receptors at the plasma membrane of target cells.

Growth Hormone Bio-Activity

GH (also referred to as somatotropin) belongs, together with placental lactogen and prolactin, to a family of pituitary polypeptide hormones that evolved from a common precursor [13]. The GH molecule contains four α-helical domains and two di-sulfide bonds. It is synthesized and stored by somatotroph cells within the anterior pituitary gland, and secreted as a 191 amino acid, 22kDa polypeptide [14]. GH synthesis in a number of extrapituitary tissues has been reported, suggesting that GH may also have local paracrine/autocrine effects [15]. GH promotes not only postnatal longitudinal

growth in children but is active throughout an individual's life in protein, fat and carbohydrate metabolism. GH is an anabolic hormone, inducing a positive nitrogen balance and protein synthesis in muscle [16]. Hyposecretion leads to dwarfism, whereas hypersecretion leads to gigantism, a clinical condition known as acromegaly. The secretion of GH is regulated by a complex neuroendocrine system that involves both neural and feedback regulatory components. The control of secretion is achieved by at least two hypothalamic hormones, a stimulatory GH-releasing hormone (GHRH) and an inhibitory hormone, somatostatin, which results in the generation of a striking pulsatile pattern of GH release [17]. Experimental animal studies have established that both growth and metabolic actions are dependent on the pattern of GH exposure, indicating that signaling is concentration-dependent and time-dependent [18]. Influences of gender, body composition, and exercise, play important roles in influencing circulating GH concentrations. Secretion declines during normal aging and many age-related changes, including osteoporosis and muscle atrophy may be due, in part, to the decreased actions of GH and IGF-1 [19].

Once in the bloodstream, GH encounters the GH-binding protein (GHBP) [20, 21], which corresponds to the extracellular domain of the GHR itself [22]. GHBP is, under normal physiological conditions, complexed with about half of the GH in human plasma [23]. By interacting with GH, GHBP forms complexes that increase the amount of GH in the circulation, thereby delaying the clearance of GH from the bloodstream and protecting it from degradation [24]. GHBP binding of GH in the circulation could also serve to allow only peak levels of GH, that exceed the binding capacity of circulating GHBP, to signal [25]. Which effects are most important is not clear and those effects that are significant may depend on many factors regulating GH and/or GHBP levels. By binding GH, the GHBP can potentiate or inhibit the GH-bio-activity and modulate GH-signaling.

The Growth Hormone Receptor

Binding of GH to the GHR represents the initial step in GH action. The variations in bio-activity of GH, are only significant if the growth hormone receptors in target cells of different tissues can discriminate these variations and transduce the appropriate signal.

The cytokine receptor superfamily

Using GH-affinity purification techniques it was possible to purify the receptor 5000-fold from rabbit liver [26]. The receptor was cloned in 1987 from rabbit liver cDNA by Leung *et al* [22] and the 3.9 kB cDNA coded for an open reading frame of 638 amino acids and a 620 residue mature form. The human sequence also coded for a 638-residue receptor which was 84% homologous. Subsequently, the rat, mouse, cow, sheep, pig, and chicken GH receptor cDNAs were cloned, revealing over 50% sequence

conservation [reviewed in 27]. The GHR is a trans-membrane protein, with an extracellular domain of 246 residues, a single transmembrane domain of 24 residues, and 350 residues intracellularly. The full-length GHR has an apparent M_r of 130.000 and contains five potential N-glycosylation sites in the extracellular domain which are not required for GH binding [28, 29].

The GHR belongs to the class I superfamily of cytokine receptors, including erythropoietin, prolactin, granulocyte colony stimulating factor, several of the interleukins, leukaemia inhibitory factor, ciliary neurotrophic factor, oncostatin M and leptin receptors. These receptors posses the following common features: limited homology (14 - 44%) in the extracellular domain, the presence of two pairs of cysteine residues and a conserved tryptophan adjacent to the second cysteine in the N-terminal fibronectin domain, a WSXWS (Trp, Ser, any amino acid, Trp, Ser) or equivalent motif in the C-terminal fibronectin domain, and two conserved membrane proximal sequences in the cytoplasmic domain, referred to as boxes 1 and 2. The extracellular domain of the GHR contains seven cysteines, of which six are linked by disulfide bonds and one, at position 241 proximal to the membrane, is free [30]. This unpaired Cys-241 is critical for GH-induced disulfide linkage but is not essential for GH-binding or signal transduction [31, 32]. The GHR possesses a variant WSXWS consensus sequence, YGEFS (Tyr, Gly, Glu, Phe, Ser), which is involved in binding of GH [33]. Members of the cytokine receptor family lack intrinsic kinase activity and utilize a group of non-receptor tyrosine kinases, known as Janus kinase (JAK), to activate signal transduction [34]. Box 1 in the cytoplasmic domain is the site for JAK2 binding and is essential for signaling [35]. The box 1 sequence is eight residues in length and located within 20 residues of the plasma membrane, it is a proline rich sequence and contains ILPPVPVP (Ile-Leu-Pro-Pro-Val-Pro-Val-Pro) in mammalian GHR [36].

Generation of the biological signal: receptor dimerization

The importance of the GHR in regulating somatic growth was demonstrated in GH-resistant Laron dwarfs that had exon deletions in the extracellular domain of the receptor [37]. Upon GH-binding, GHR mediates proliferation in the interleukin-3-dependent FDC-P1 and Ba/F3 cells [38, 39], insulin production in rat insulinoma (RIN-5A) cells [40, 41], and protein and lipid synthesis in CHO cells [42, 43]. In response to GH, the tyrosine kinase JAK2 rapidly associates with the receptor, is activated and becomes tyrosyl phosphorylated [35, 44]. Activation of JAK appears to be a common signaling event that occurs in response to ligand binding to members of the cytokine receptor superfamily. Several studies performed on the extracellular domain of the GHR, using crystallography and size-exclusion chromatography, have suggested that one GH molecule binds to two receptors [45, 46]. This binding was hypothesized to occur sequentially, in which the first receptor binds with high affinity to GH binding site 1 while contacts at site 2 and an contact region between the two extracellular domains of the GHR

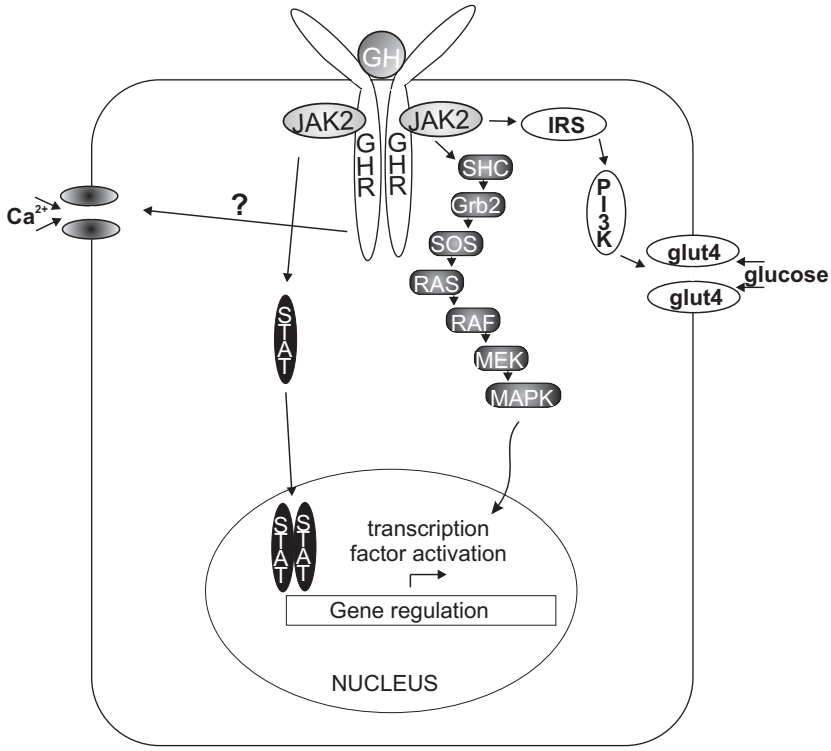
stabilize the binding of the second receptor [47]. The use of a GH antagonist mutated at GH binding site 2 (G120R) indicated that GHR dimerization is essential for GH-stimulated cell proliferation [48].

A GH molecule that is capable of dimerizing GHRs at the cell surface is not sufficient for signal transduction. That, besides dimerization, a conformational change is needed for a biological signal, was first suggested by Mellado *et al* who developed a specific monoclonal antibody that recognized the hinge region of the extracellular domain of the GHR. Antibody binding to the cell surface receptor increased upon GH binding, but not when the GH antagonist GH(G120R) was used [49]. Further support for specific conformational changes for signaling came from a study by Rowlinson *et al*, who found that of 14 tested mAbs to the GHR, only two served as weak agonists in a GHR-based proliferation assay, while eight were able to dimerize the receptor [50].

Signal-transduction

In response to GH, the non-receptor tyrosine kinase JAK2 associates with the dimerized complex, is activated, and tyrosyl phosphorylated [35, 44, 51]. JAK2 is a member of the Janus family of cytoplasmic tyrosine kinases that include JAK1, JAK2, JAK3 and tyk2 [52, 53]. Using mutated box 1 regions of the GHR, it was shown that the proline-rich domain is intermediate in JAK2 binding and that, in response to GH binding, the affinity of JAK for the GHR appears to increase [38, 54]. Receptor dimerization brings two JAK2 molecules in close proximity, each kinase then *trans*-phosphorylates one or more tyrosines in the kinase domain of the paired JAK2. Activation of JAK2 is rapid and transient, resulting not only in the tyrosyl phosphorylation of the GHR and the kinase itself, but also leading to a cascade of phosphorylation of cellular proteins [55-57]. Phosphorylated tyrosines have the potential to serve as docking sites for Src homology 2 (SH2) or phosphotyrosine binding (PTB) domain containing proteins, thereby activating specific signaling pathways [58]. One subset of activated proteins is the signal transducer and activator of transcription (STAT) family of transcription factors that couple ligand binding to cellular receptors with the activation of gene transcription [59]. Stat proteins associate with the activated GHR and/or JAK2 through their SH2 domain and are phosphorylated on their conserved C-terminal tyrosine, they complex with other stat proteins, translocate to the nucleus, bind to DNA and activate transcription of target genes [reviewed in 60]. Two other proteins that are rapidly phosphorylated upon GH stimulation, are the MAP kinases ERK-1 and ERK-2. One of the pathways leading to the MAP kinases involves Shc, growth factor receptor-bound-2 (Grb-2), son of sevenless (SOS), Ras, Raf and mitogen-activated/extracellular signal-regulated kinase (MEK) [61, 62]. MAP kinases, family of the serine/threonine/tyrosine kinases, are activated by a number of receptor and non-receptor kinases and are believed to be important regulators of cellular growth and differentiation [63]. GHR activation also stimulates the rapid tyrosyl phosphorylation of insulin receptor substrate (IRS)-1 in primary

Figure 1. Schematic representation of GH/GHR intracellular signaling pathways



In response to GH, JAK2 is activated and tyrosyl phosphorylated. The activated JAK2 phosphorylates the GHR and downstream signaling molecules. A JAK2-independent signaling pathway has been suggested in GH-inducible calcium influx. For details of the mechanisms and abbreviations, see the text.

cultures of rat adipocytes [64], and in 3T3-F442A fibroblasts [65]. Many of the metabolic effects of GH may be mediated via the IRS molecules [64]. IRS-1 and IRS-2 function as docking proteins after phosphorylation and associate with the 85 kDa regulatory subunit of PI-3-kinase and the tyrosine phosphatase SHP-2 [66]. 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 plasmamembrane that ultimately stimulates glucose uptake.

Splice defects in the extracellular region of the GHR in patients with Laron dwarfism provided strong proof for the functional significance of the GHR [37]. The Laron syndrome, which is characterized by inherited GH insensitivity (GHIS), was identified by Laron *et al* and has a clinical phenotype of severe growth retardation with high circulating GH, accompanied by low serum IGF-1 and IGF-binding protein-3 (IGFBP-3), and no responsiveness to exogenous GH [67]. In the inherited GHIS, over 30 mutations in the GHR have been described, with the majority of the mutations in the

exons that code for the extracellular domain of the receptor, interfering with GH binding [68, 69]. Recently, a heterozygous point mutation in the splice acceptor site, upstream of exon 9, was described. This mutation resulted in exon 9 being omitted from the GHR mRNA, creating a truncated receptor GHR(1-277) with only seven residues in the intracellular domain [70, 71]. Alternative splices of the GHR, resulting in truncated isoforms, were identified in a permanent cell line of IM-9 cells and in a number of human tissues [72, 73]. The alternative splices represented less than 10% of the total transcripts and are believed to act as dominant negative inhibitors of GHR-signaling by heterodimerization with the full-length receptor. These findings suggest that differential expression of GHR isoforms could play a significant role in GHR signaling.

Signal down-regulation

GH-induced signaling is terminated as a result of the initiation of negative regulatory pathways after activation of the GHR. The molecular mechanism of JAK deactivation is still poorly understood. One mechanism that contributes to signal down-regulation is the activation of tyrosine phosphatases, enzymes involved in the dephosphorylation of JAK2. The phosphatase SHP-1 interacts with JAK2 and GH stimulates its catalytic activity [74]. The transmembrane glycoprotein signal-regulatory protein (SIRP)α1 can act as a negative regulator of GH-signaling by binding SHP-2 [75]. Another negative regulatory pathway of GH signaling involves the suppressor of cytokine signaling (SOCS) proteins [76, 77]. The SOCS family consists of eight proteins: SOCS 1-7 and CIS (cytokine-inducible SH2-domain-containing protein), each of which contains a central SH2-domain and a C-terminal SOCS-box [reviewed in 78]. SOCS proteins act as negative regulators of cytokine signaling, part of a classical negative feedback loop, since their expression is induced in response to stimulation with cytokines. The ubiquitin-proteasome pathway is the third mechanism involved in the (down-)regulation of GHR signal tranducing events [79]. Down-regulation of GH-signaling requires the activity of the proteasome but was found to be independent of GHR endocytosis. Proteasome inhibitors prolong the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation [80]. A GHR box 1 mutant, unable to associate with JAK2, demonstrates that GHR internalization and signal transduction are independently regulated by the ubiquitin-proteasome pathway [81].

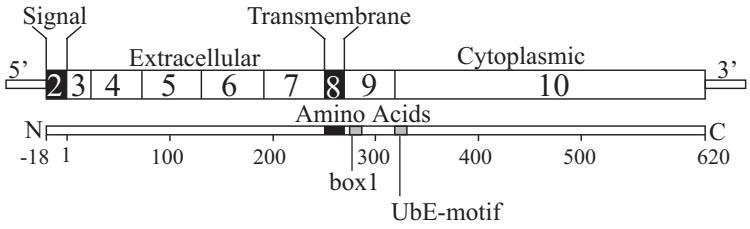
Regulation of the GHR

Target cells are only responsive to GH if they express sufficient functional GHRs at their cell surface. The GHR life cycle is mainly determined by three processes: 1. Receptor biosynthesis and transport to the cell surface, 2. GHR proteolysis and shedding of the extracellular domain as GHBP, and 3. GHR internalization and subsequent lysosomal degradation. Together, these processes determine the surface expression or *availability* of the receptor for its ligand.

The human GHR gene, localized to the proximal short arm of chromosome 5, comprises at least ten exons, extending over 87 kb, with the receptor itself being encoded by nine exons [Figure 2, and 37]. Exon 2 encodes a secretion signal sequence, exons 3 through 7 encode the extracellular GH binding domain, exon 8 encodes a transmembrane domain and exons 9 and 10 encode the cytoplasmic tail and a 3' untranslated region [reviewed in 69]. GHRs are most abundant in liver but are widely expressed in human tissues including, adipose tissue, muscle, kidney, heart, brain and more [82]. In general, the expression of GHR increases considerably after birth, reaching a plateau by adulthood. Tissue-specific control of receptor expression is based on alternate exon 1 splicing; the existence of up to seven exon 1 promoters for the GHR provides great potential flexibility in the control of receptor expression. The GHR is synthesized in the endoplasmic reticulum (ER), processed in the Golgi and transported to the plasma membrane. The receptor turnover is rapid with reported half-lives of 45 to 90 min. Hormone accelerated receptor down-regulation is observed in IM-9 lymphocytes [83, 84], in cultured rat adipocytes [85] and in mouse fibroblasts [86] but not in freshly isolated rat adipocytes [87].

Proteolysis of GHR at the cell surface, leading to shedding of GHBP is one mechanism which contributes to receptor turnover in rabbits and humans [88]. In rodents (rat and mouse), the GHBP is secreted as an alternative splicing product of the GHR gene that contains a short hydrophilic C-terminal extension that is not found in the membrane receptor [89, 90]. This hydrophilic tail is encoded by a special exon (8A) interposed between exons 7 (extracellular) and 8 (transmembrane domain) [91]. In many other species exon 8A is missing, and the GHBP is generated by proteolytic cleavage from the membrane-bound GHR, a process also known as shedding. Shedding of GHBP was observed in cell culture model systems and it was shown that such processing could be induced with phorbolesters, growth factors and the alkylating reagent N-ethylmaleimide (NEM) [92]. It is suggested that the MAP-kinase pathway is involved in the phorbolester-induced, protein kinase C (PKC) dependent activation of GHR proteolysis [93]. Activation of proteolysis was proposed to involve regulation of the activity of

Figure 2. Schematic representation of the GHR



The GHR gene comprises ten exons. Nine exons encode for a 620 amino-acid protein. Exons 3 through 7 encode the extracellular GH-binding domain, exon 8 encodes the 24 amino-acid transmembrane domain, and exons 9 and 10 encode the cytoplasmic tail.

the secretase by a cysteine-switch mechanism and/or PKC dependent phosphorylation [94]. Recently, it was shown that the transmembrane metzincin metalloprotease, tumor necrosis factor-α-converting enzyme (TACE or ADAM-17) can function as a GHR secretase and that shedding could be inhibited with metalloprotease inhibitors [95]. Phorbolester-induced GHR proteolysis, results in down-regulation of GHR abundance and GH-signaling and is inhibited after receptor dimerization by GH [96].

Internalization is a second pathway for removal of the GHR from the cell surface, and ongoing protein synthesis is important to maintain binding capacity [97]. The GH-GHR complex internalizes via clathrin-coated pits [83, 98, 99] and is transported to the lysosomes to be degraded [100, 101]. Dissociation of the GH-GHR complex does not occur at (endosomal) pH 5.5 [102] and lysosomal GH degradation is inhibited by agents that interfere with the endosomal pH, such as ammoniumchloride and chloroquine [86]. Transport of GHR to the nucleus [103], to detergent-insoluble membrane domains [104], and back to the plasma membrane [105] has also been reported. Recently, it has become apparent that the ubiquitin-system is involved in endosomal trafficking of membrane receptors, transporters and channels [reviewed in: 106, 107]. Several mammalian receptor proteins, such as epidermal growth factor (EGF) receptor [108], platelet derived growth factor (PDGF) receptor [109], *c-kit* receptor [110], T-cell receptor [111] and the Met receptor [112] are ubiquitinated in response to ligand binding. Most proteins that are ubiquitinated at the plasma membrane are targeted for degradation in the lysosome. The GHR was initially found ubiquitinated upon purification of the receptor from rabbit liver [22]. Binding of GH to its receptor stimulates ubiquitination, internalization and degradation of the receptor. Using a Chinese hamster cell line carrying a temperature sensitive ubiquitin-activating enzyme (E1), it was shown that inactivation of E1 results in an accumulation of non-ubiquitinated GHRs at the cell surface, while endocytosis of transferrin is unaffected [113]. Ligand-induced endocytosis of the GHR is mediated by the ubiquitin system via a 10 amino acid motif within the cytoplasmic tail (UbE motif; DSWVEFIELD) but ubiquitination of the receptor itself is not required [114].

The Ubiquitin-Proteasome Pathway

The 2000 Albert Lasker Award for Basic Medical Research was presented to Aaron Ciechanover, Avram Hershko and Alexander Varshavsky for the discovery and recognition of the broad significance of the ubiquitin system in regulated protein degradation. Ubiquitin-mediated proteolysis plays a major role in many basic cellular processes. Among these processes are regulation of the cell cycle, differentiation and development, the cellular response to extracellular effectors and stress, modulation of cell surface receptors and ion channels, DNA repair and regulation of the immune and inflammatory responses. In most cases, ubiquitin modification targets the substrate for degradation by the 26S proteasome, but in certain cases, ubiquitin modification leads to targeting to the lysosome/vacuole [reviewed in 115-119].

Regulation by degradation

Proteins, as the regulators of cellular function, exist in a dynamic state. Protein levels reflect a delicate balance between production and destruction. Although early work focused on the production of proteins as the main regulator of protein levels, the work from Schoenheimer in the 1950's showed that proteins also have a time to die. This work laid the foundation for the discovery that intracellular protein degradation is tightly controlled and highly regulated. Some proteins in cells remain stable for long periods of time, while others disappear rapidly. At present there are two predominant cellular systems for the degradation of cellular proteins: the vacuolar pathway (including lysosomes and endosomes) and the cytosolic ubiquitin-proteasome pathway. Ubiquitin is a 76-amino-acid globular protein of 8.5 kDa that is highly conserved throughout eukaryotes. The classical view of the ubiquitin-proteasome pathway is that it targets proteins for degradation in two distinct and successive steps: 1) covalent attachment of multiple ubiquitin molecules to the protein substrate, and 2) targeting of such a tagged protein for degradation by a multisubunit, ATP-dependent protease, termed the proteasome. Conjugation of ubiquitin to the substrate requires the sequential action of three enzymes. The C-terminal Gly residue of ubiquitin is activated in an ATP-requiring step by a specific activating enzyme (E1). This step consists of an intermediate formation of ubiquitin-adenylate, followed by the binding of ubiquitin to a Cys residue of E1 in a thiolester linkage, with the release of AMP. Activated ubiquitin is next transferred to an active site Cys residue of one of several ubiquitin-carrier proteins (E2, ubiquitin conjugating enzyme, Ubc). Finally, the last step in the conjugation process is catalyzed by a ubiquitin-protein ligase (E3) which links ubiquitin by its C-terminus in an amide isopeptide linkage to an ε-amino group of an internal Lys residue of the protein substrate. Recently, a new class of ubiquitination enzymes, E4, has been identified. [120]. E4 is in some cases required for the polyubiquitination of monoubiquitinated substrates. In successive reactions, a polyubiquitin chain is synthesized by transfer of additional activated ubiquitin moieties to lysine residues of the previously conjugated ubiquitin molecule. This polyubiquitin chain serves as a recognition marker for the 26S proteasome. Ubiquitination is a dynamic and reversible process and deubiquitinating enzymes (DUBs, isopeptidases, ubiquitin C-terminal hydrolases UCHs) cleave ubiquitin from proteins and disassemble multi-ubiquitin chains. DUBs have the potential to either accelerate proteolysis or inhibit it. There are at least 19 yeast DUBs and substantially more in mammals, suggesting that some of them may have specific functions, such as recognition of distinct tagged substrates.

E1: ubiquitin activating enzyme

The ubiquitin-activating enzyme E1 (Uba in yeast) is the product from a single gene with two isoforms of 110 kDa and 117 kDa, arising from alternative translation start sites [121]. Deletion of the E1 gene is lethal and the finding that cells expressing a tem-

perature-sensitive E1 undergo cell-cycle arrest provided the first evidence for the physiological significance of ubiquitination [122].

E2: ubiquitin-carrier protein

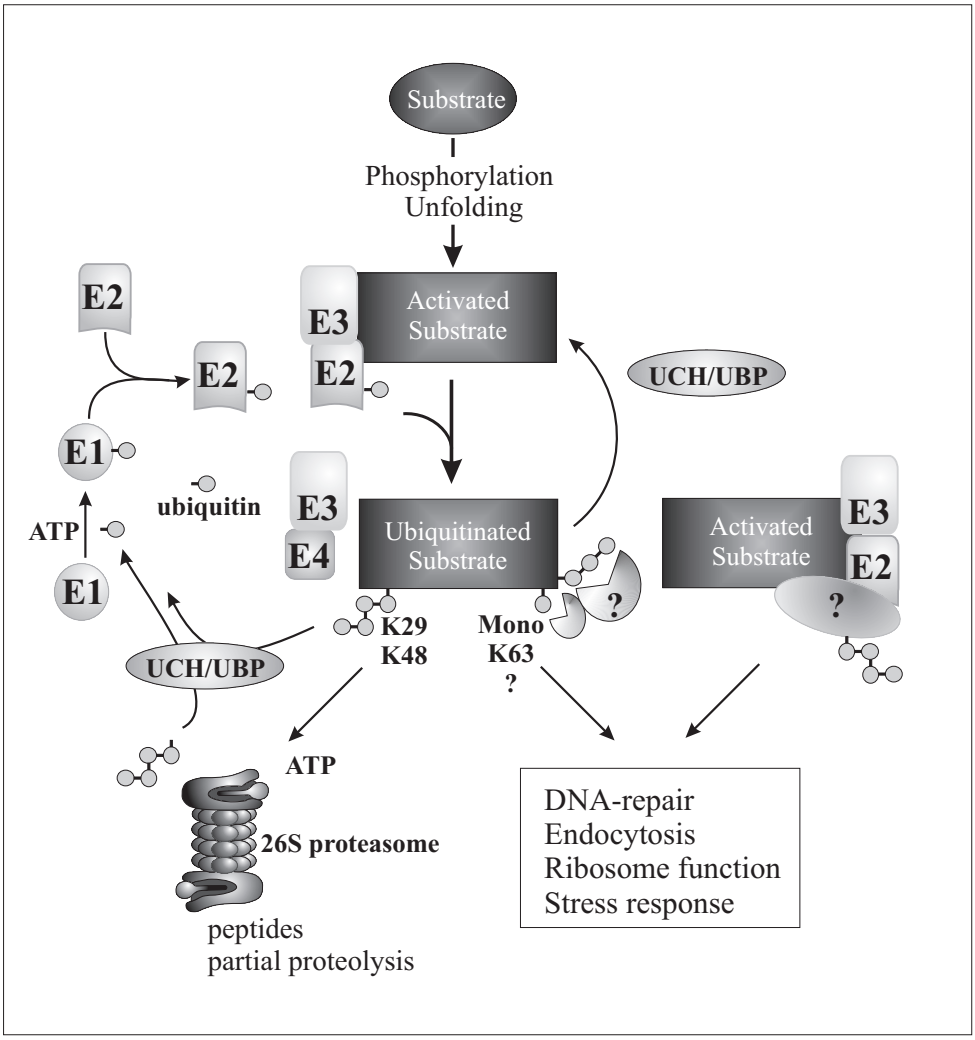
The specificity of the system becomes apparent in the large family of E2s; 13 E2-like genes have been identified in *S. cerevisiae*, termed Ubc1-13. Structurally, all known E2s share a 14-16 kDa core domain that is ~35% conserved among family members. This domain contains the active site Cys residue required for the formation of the ubiquitin thiolester linkage [123]. Most known E2s are 14 - 35 kDa, several are limited to the core domain while others have significant N-terminal or C-terminal extensions which may facilitate interactions with E3s or serve as membrane anchors and contribute to the cellular localization [124, 125]. The specific functions of most E2s are due to their association with distinct E3s, which in turn, bind specific protein substrates. Many E2s act in concert with more than one E3 enzyme, for instance human UbcH7, can interact with both the HECT-domain of E6-AP and the RING finger of c-Cbl. In addition, some E3 enzymes act with several E2s [126, 127].

E3: ubiquitin-protein ligase

An E3 enzyme is defined as a protein that binds the target substrate, either directly or indirectly via ancillary proteins, and catalyzes transfer of ubiquitin from a thiolester intermediate on E2 or E3 to an amide linkage with the substrate or with a polyubiquitin chain already anchored to the substrate. Since the target proteins bind to the ligases prior to conjugation, E3s are the key players in determining the high specificity of the system. Two distinct E3-families containing conserved protein domains have been identified. HECT (homologous to E6-AP C- terminus) domain E3s form thiol-ester intermediates with ubiquitin as part of the process, leading to ubiquitination of substrates [128]. Members of the other class, Really Interesting New Gene (RING)-finger E3s, are now believed to mediate the direct transfer of ubiquitin from E2 to the substrate [129].

E6-AP (E6-associated protein) is the founder of the HECT-domain family of E3s. This ligase was found to be required, along with the human papillomavirus (HPV) E6 oncoprotein, for the ubiquitination and degradation of the tumor suppressor protein p53. E6 serves as an adaptor between E6-AP and p53, allowing E6-AP to catalyze the ubiquitination of p53. The HECT-family proteins are closely related to E6-AP in a ~350 residue region at their C- terminus, the HECT domain, which includes the ubiquitin binding Cys residue. A feature shared by many HECT E3s, is the WW-domain, which is involved in protein-protein interactions and plays a role in targeting substrates for ubiquitination [130]. Rsp5p is one of the five HECT-E3s in the yeast *S. Cerevisiae* and it exemplifies the capacity of a single E3 to ubiquitinate distinct proteins in several cellular compartments. Rsp5p is best known for its ability to ubiquitinate at least 13 plasma membrane proteins and transporters [131]. Nedd4 is another HECT-domain E3, which binds and ubiquitinates subunits of the epithelial sodium channel (ENaC)

Figure 3. Schematic representation of the ubiquitin-proteasome pathway



Cellular events like, e.g. phosphorylation, lead to activation of the substrate. The activated substrate is conjugated to ubiquitin through the sequential action of E1, E2 and E3. In some cases, a multiubiquitination factor (E4) is required to drive polyubiquitin chain assembly. Polyubiquitin chains linked through K48 or K29 of ubiquitin are the targeting signals leading to degradation by the 26S proteasome. In two cases limited cleavage of substrates by proteasomes has been shown.
Deubiquitinating enzymes (UCH/UBP) can remove ubiquitin from ubiquitinated substrates and prevent their degradation or release free ubiquitin from polyubiquitin precursors and restore the cellular ubiquitin pool. Monoubiquitination is sufficient for endocytosis of some substrates. K63-linked polyubiquitin chains function in DNA-repair, ribosome function, stress response or endocytosis. The molecular mechanisms involved are unresolved. In some cases, ubiquitination of the substrate itself is not required, indicating that ubiquitination of other factors may be involved. Proteins that recognize monoubiquitinated substrates or K63-linked polyubiquitin chains have not been identified.

through its WW domains leading to down-regulation of the number of active channels [132].

RING finger E3 ligases are proteins that contain a zinc-stabilized RING finger motif that binds to E2 and a domain that binds to the protein substrate to be degraded [133]. The RING-finger was described in the early 1990s, years before a role in ubiquitination was suspected. In the past few years a number of investigations led to the realization that the RING-finger plays a general role in ubiquitination. First, it was discovered that a small RING finger protein, Rbx1 (Ring box protein-1, ROC1, Hrt1), was a requisite component of the multisubunit family of E3s, SCF (Skp1/Cul1/F-box protein) [134, 135]. Later studies acknowledged that many otherwise unrelated RING finger proteins mediate ubiquitination [136]. Although there is no clear answer yet, covalent linkages between ubiquitin and RING finger proteins are unlikely to exist. It is now believed that RING E3s may serve as scaffolds that position and orient the protein substrate and E2 optimally for ubiquitin transfer. RING finger ligases can be divided into single-subunit E3s, containing the substrate recognition element and the RING finger on the same polypeptide, and multisubunit E3s. Multisubunit E3s include a small RING finger protein and a member of the Cullin family of proteins as well as other proteins, some of which recognize substrates. Single subunit RING finger proteins include Mdm2, which ubiquitinates p53 [137, 138], the protooncoprotein c-Cbl, which ubiquitinates growth factor receptors [139-141], and the inhibitors of apoptosis (IAPs) [142]. Multisubunit, cullin containing RING E3s, include the SCF complex, the VHL-CBC (von Hippel-Lindau-Cul2/Elongin B/Elongin C) complex and the anaphase promoting complex (APC) [143, 144]. The SCF class of ubiquitin ligases contain at least four proteins: Skp1, Cul1, Rbx1 and an F-box protein. SCF substrates are bound directly by adaptors called F-box proteins. F-box proteins contain a ~45 residue motif (F-box) which mediates Skp1 binding and bind to substrates through protein-protein interaction domains [145]. There are at least 19 F-box proteins in *S. cerevisiae*, over 100 in the *C. elegans* genome, and ~50 described so far in vertebrates. In the highly studied SCF complexes SCF^{CdC4}, SCF^{Grr1}, SCF^{Skp2} and $SCF^{\beta\text{-}Trcp}$ (the superscript denotes the identity of the F-box subunit), substrate phosphorylation appears to precede F-box protein binding [146]. The β-transducin-repeat-containing protein (β-TRCP) is an example of an F-box protein that recognizes a specific phosphoserine motif (DSGXS) in more than one phosphorylated substrate (β-catenin and IκBα). One architectural relative to the SCF E3s is the VHL-CBC complex. It contains Rbx1, the cullin Cul2, a protein homologous to Skp1 called elongin C, a probable adaptor protein called von Hippel Lindau (VHL), and another factor called elongin B. VHL binds to elongin C through a special motif, the BC box. This motif is found in a number of proteins, including VHL and members of the SOCS-family, where it is embedded within a "SOCS-box" domain. The BC box is necessary and sufficient for VHL, SOCS1 and SOCS 3 to bind to elongin C [147, 148]. The BC-box/elongin C interaction might parallel the F-box/Skp1 interaction. VHL downregu-

lates hypoxia-inducible mRNAs by controlling proteolysis of hypoxia-regulated transcription factors Hif1α and Hif2α, which positively regulate vascular endothelial growth factor (VEGF) [149, 150]. The most complicated of the multisubunit E3s is the anaphase-promoting complex (APC), which was the first multicomponent ubiquitin ligase described. In *S. cerevisiae*, at least 12 essential APC components have been described. The APC contains a cullin homologue (Apc2) and a RING finger protein similar to Rbx1, called Apc11. The protein substrates for this E3 include mitotic cyclins, some anaphase inhibitors, and spindle associated proteins, all of which are degraded at the end of mitosis. Two destruction signals have been identified in substrates targeted for degradation by the APC, the destruction box (D-box), and the KEN box [151, 152]. The nine-residue D-box is found in all the known substrates of the APC using the Cdc20 adaptor (APC^{Cdc20}). Recently, the KEN-box was described as a transposable, seven-residue motif that targets substrates to the APC^{Cdh1}. Phosphorylation and dephosphorylation are known to be important regulators of APC-activity [153].

E4: multiubiquitination factor

Recently, an additional conjugation factor that facilitates the formation of multiubiquitin chains is identified and named E4 [120]. E4 binds to a monoubiquitinated substrate and drives multiubiquitin chain assembly, in conjunction with the E1, E2 and E3 enzymes. E4 defines a novel protein family, which includes the yeast protein UFD2 and contains a highly conserved C-terminal domain, termed the U-box (UFD2-homology domain). E4 activity requires the presence of the relevant E3-ligase but is not in all cases required for the formation of multiubiquitin chains. Recently, overexpression of two novel components, Bul1p and Bul2p, which form a complex with the E3-ligase Rsp5p, was shown to increase the number of ubiquitin moieties added to Gap1p. These results suggest that Bul1p and Bul2p equip Rsp5p with E4 activity [154].

Deubiquitinating enzymes (DUBs)

Cells contain many deubiquitinating enzymes, cysteine proteases that cleave ubiquitin molecules that are conjugated via either isopeptide or linear peptide bonds. Deubiquitination is essential for the maturation of newly synthesized ubiquitin molecules which are often translated as a linear, “head” to “tail”, polyubiquitin molecule. Deubiquitination is also essential for the release of ribosomal proteins that are synthesized fused at their N-terminal residue to a ubiquitin moiety that targets them to the ribosome and for the recycling of ubiquitin molecules after degradation of the substrate [reviewed in 155, 156]. The DUBs are divided into two classes: the ubiquitin C-terminal hydrolase (UCH) family and the ubiquitin-specific processing protease (UBP) family, both of which are thiol proteases. The UCH family enzymes are ~25 kDa proteins that catalyze the removal of C-terminal fusion proteins from ubiquitin. They selectively cleave off small leaving groups and/or extended peptide chains from the C-terminus of ubiquitin. UBPs are generally larger, ~100 kDa, and are considered responsible for

removing ubiquitin from either conjugates with cellular proteins or from free polyubiquitin chains. A large number of DUBs are encoded by both yeast and higher eukaryotic genomes, suggesting that some of them may perform specific functions. The *Drosophila melanogaster Fat Facets (FAF)* gene encodes a deubiquitinating enzyme that was shown to be involved in photoreceptor development [157]. DUBs can either stimulate or inhibit proteolysis. By removing ubiquitin from tagged proteins, they prevent degradation of the target protein by counteracting the formation of polyubiquitin chains. Stimulation of proteolysis can be mediated by the release of free ubiquitin from biosynthetic precursors thereby restoring the cellular ubiquitin pool. A new family of DUBs, related to cytokine signaling, was identified in T-lymphocytes. DUB1 and DUB2 are induced by stimulation of the cytokine receptors for IL-3, IL-5, and GM-CSF. This suggests that regulation of these DUBs is part of the cellular response to the JAK-STAT signal transduction pathway [158].

The proteasome

Most polyubiquitinated proteins contain K48-linked chains and are recognized by the 26S proteasome as targets for degradation. The 26S proteasome is composed of the 20S catalytic core complex and is capped on both sides by a 19S multisubunit regulatory complex. Generally, proteasomes degrade proteins into small peptides of about 8 amino acids which can be hydrolyzed to amino acids by cytosolic peptidases [reviewed in 159, 160]. The 20S proteasome is a cylindrical chamber composed of a stack of four rings, each ring containing seven subunits [161]. The outer rings consist of α-subunits, while the two inner rings are composed of β-subunits. The three catalytic sites: the trypsin-, chymotrypsin- and post-glutamyl peptidyl hydrolytic-like sites, reside on the β- subunits [162]. The 19S cap, or regulatory complex, contains two multisubunit components, the “base” and the ”lid”. The base, which is composed of six ATPases of the triple-A family plus two non-ATPase subunits, binds to the 20S catalytic core. The ATPases carry out a chaperone-like activity and are believed to help unfold substrates and channel them into the 20S core, thereby controlling access of substrates to the catalytic chamber. The residual eight different non ATPase subunits of the 19S regulator form the lid. The exact function of these components is unknown [163]. One of the functions of the 19S cap is to recognize the polyubiquitin signal on the target protein, so that intracellular proteins are not indiscriminately destroyed.

Although the proteasome is thought to degrade the targeted substrates completely and in a ubiquitin-dependent way, exemptions have been reported. Limited cleavage of substrates by proteasomes has been demonstrated in the case of the activation of two transcription factors, Spt23 and Mga2. The cleavage of the soluble components of these transcription factors from their ER-membrane bound precursors was ubiquitin- and proteasome dependent [164]. Limited processing of the precursor protein p105 in p50 by the proteasome has also been reported in the case of the maturation of transcription fac-

tor Nuclear Factor κ-B (NF-κB). The p50 protein can interact with RelA (p65) to form the active NF-κB [165-167]. Ubiquitin independent proteasomal targeting has been reported in the case of ornithine decarboxylase and $p21^{cip1}$ [168, 169].

Degradation of ER-retained (membrane)-proteins

The components of the ubiquitin system reside in the cytosol and the nucleus and most of the identified physiological substrates for proteasomal degradation are cytosolic or nuclear proteins. In the past few years research has shown that also membrane-anchored proteins and even endoplasmic reticulum (ER) lumenal proteins can be modified and degraded by the ubiquitin system [reviewed in: 170, 171]. The ER is the site of synthesis and serves as a quality control center for proteins destined for secretion, for the plasma membrane, and for endocytic organelles. Proteins are inserted into the ER membrane or imported into the lumen in an unfolded state. Proper folding of these proteins in their native conformation is an important function of the ER, to assure that only biologically active proteins reach their destination. The ER is equipped with a highly efficient quality control (QC) system that guarantees that only properly folded proteins are delivered. Misfolded and incompletely assembled proteins are retained in the ER and eventually degraded in a process that is called ER-associated degradation (ERAD). The unassembled proteins are recognized by ER chaperones such as calnexin (Cne1p) or Bip (Kar2p) and, if not properly folded or assembled, are retrotranslocated through the Sec61 channel into the cytosol, polyubiquitinated and degraded by the proteasome. Many basic aspects of this complex process remain obscure, especially those that involve the selective identification of the substrates and the mechanism of retrograde translocation across the ER membrane back into the cytosol. In yeast, mutant versions of the carboxypeptidase Y (CPY*), the plasma membrane ATP-binding cassette transporter Pdr5p (Pdr5*), the ER membrane-located Sec61p, the mating type pheromone α-factor, and down-regulated hydroxymethylglutaryl(HMG)-CoA reductase were found to be subject to ER degradation [172-175]. Studies on CPY* and Pdr5* revealed that polyubiquitination of these mutant proteins depends on the ubiquitin conjugating enzymes Ubc6 and Ubc7 [170]. Ubc6 was identified as an ER-membrane bound component of the yeast ubiquitin system, with the active site residing at the cytoplasmic face of the ER-membrane [125]. The ER-membrane protein Cue1p is required for recruitment of Ubc7p to the ER-membrane and for its activation [176]. Genetic screens have identified a variety of gene products in the ER-membrane which are necessary for ubiquitination and degradation of the respective proteins [177]. Der1p was shown to be necessary only for ER-degradation of soluble fully translocated CPY* [178]. The identification of a genetic interaction between Der3/Hrd1p, Hrd3p and Sec61p has led to the proposal that these proteins formed both the retrotranslocon and an E3 complex [179]. Recently, it was shown that Der3/Hrd1p is a RING domain-containing protein which resides in the ER membrane, uncovering this protein as the E3 ligase of the ER-associ-

ated degradation process [180]. In mammalian cells, substrates that undergo ER-associated degradation are unassembled T-cell receptor (TCR) α-chains and a mutated version of the human cystic fibrosis transmembrane conductance regulator (CFTR) [181-184]. In T-lymphocytes polyubiquitinated forms of TCR α and the TCR subunit CD-3δ are associated with the ER membrane, suggesting that their ubiquitination occurs while still membrane bound [185]. Recently, two murine homologs of Ubc6 and Ubc7 were characterized. MmUbc6 is an integral membrane protein that is anchored via its hydrophobic C-terminal tail to the ER. MmUbc7, which is not an integral membrane protein, shows significant ER-colocalization with MmUbc6 [186]. These findings implicate a specific E2 in degradation from the ER in mammalian cells. The E3-ligase involved in ERAD in mammalian cells is not identified, but the search for the mammalian homolog of the yeast Hrd1p/Der3p is now the obvious objective to pursue. Incorrectly folded proteins are not the only targets for the ubiquitin-proteasome-catalyzed ER degradation pathway. Regulated degradation of ER-membrane proteins also follows this route, as is the case with HMG-CoA reductase, a protein whose activity is regulated by ERAD. In yeast, two HMG-CoA reductase isoenzymes that constitute key regulatory enzymes in sterol synthesis reside in the ER membrane. The ubiquitin-proteasome pathway is responsible for the end product-induced degradation of the HMG-CoA reductase-2 isoenzyme [175].

Regulation by modification

The covalent modification of target proteins by the attachment of ubiquitin as a signal for degradation by the 26S proteasome is the well documented, classical view of the ubiquitin-proteasome pathway. An exciting development, derived from recent reports in a number of different laboratories, indicated that ubiquitin modification may play a more general role as an intracellular signal. It now appears that distinct (poly)ubiquitin signals can act in different cell processes and are created by a variation in the type of lysine linkage between the ubiquitin moieties [187, 188]. Ubiquitin itself contains seven conserved lysine residues, all of which are potential sites of isopeptide linkage to the C-terminal glycine of another ubiquitin. All seven lysine residues are capable, *in vitro*, of linking together to form chains, and linkages between K11, K29, K48 and K63 have been detected *in vivo* [189]. K48-linked polyubiquitin chains are the targeting signals that lead to recognition and degradation by the 26S proteasome. Genetic experiments in yeast, transfected with ubiquitin in which the seven lysines were individually mutated, and the outcome determined in cells with deleted genomic ubiquitin, showed that only mutations of K48 produced a dramatic effect on proteolysis [190, 191]. Mutations of lysine residue 63 indicate that K63-linked chains may be involved in distinct biological processes. Yeast strains that were blocked for chain assembly through K63, demonstrated defects in post-replicative DNA repair [191], the stress response [192], mitochondrial DNA inheritance [193], endocytosis of some plasmamembrane proteins [194],

and ribosomal function [195]. Since mutations of the other lysine residues caused no obvious phenotypes, the possible functions of polyubiquitin chains linked by these residues remain elusive. Hofmann and Pickart identified a mammalian E2 enzyme, specifically involved in the formation of K63-linked polyubiquitin chains [196]. The gene encoding this E2 protein is the ortologue of the budding yeast gene *UBC13.* The Ubc13 protein can not form chains by itself but needs the association with Mms2 or UEV1. Both proteins, Mms2 and UEV1, are members of the ubiquitin-conjugation enzyme variant (UEV) family. Members of the UEV-family of proteins bear striking similarity to the E2 ubiquitin conjugating enzymes, but lack the active site cysteine. *Mms2* and *Ubc13* mutants were shown to be defective in post-replicative DNA repair, but the role of K63-linked polyubiquitin chains is unclear. Deng *et al* showed that assembly of K63-linked polyubiquitin chains is necessary for IκB kinase (IKK) activity [197]. Activation of IKK requires Traf6 (tumor necrosis factor (TNF)-receptor-associated factor 6), which is a RING finger domain protein. Traf6 functions as the E3 ligase, that together with the dimeric ubiquitin-conjugating enzyme (E2) complex composed of Ubc13 and Mms2 (UEV1A), mediates the assembly of K63-linked polyubiquitin chains required for IKK activation. Although the actual identity of the proteins that are K63-ubiquitin modified remain unknown, this modification turns out to be a novel, non-proteolytic, function for ubiquitin which in this case regulates the activity of a protein kinase. It is likely that other enzymes and processes may also be regulated in this way.

Monoubiquitination is another example of the functional diversity of the ubiquitin signal. Protein modification by a single ubiquitin is thought to regulate processes that range from membrane transport to transcriptional regulation [reviewed in 107]. The mechanism by which monoubiquitination regulates its substrates is unknown. Ubiquitin may alter the conformation or oligomeric state of a protein, the addition of ubiquitin could recruit or inhibit binding of regulatory proteins, or ubiquitin may affect the localization of target proteins.

Histones were the first proteins found to be monoubiquitinated [198-200] but only recently, functions have been identified for histone ubiquitination [201, 202]. Histones H2A and H2B form part of the core structure of nucleosomes. Yeast cells carrying a mutant H2B that lacks its ubiquitination site do not sporulate, suggesting that H2B monoubiquitination is needed for meiosis.

Regulation of the activity of proteins located at the plasma membrane is another function for monoubiquitination in yeast. Many plasma membrane proteins are downregulated by internalization into the endocytic pathway, and in *Saccharomyces cerevisiae* most of these proteins require ubiquitination of the cytoplasmic domains to be internalized [reviewed in 130, 131]. Several studies showed that monoubiquitination is sufficient to trigger endocytosis of plasma membrane proteins [203-206] and in other cases,

di-ubiquitin chains linked through K63 further enhance the basal internalization rates [194, 207]. It is thought that the internalization information is carried in the ubiquitin polypeptide itself because in-frame fusions of ubiquitin, stimulate internalization of heterologous proteins that normally do not internalize [204, 208, 209]. Proteins of the endocytic machinery that recognize the internalization signal in ubiquitin are not identified. Monoubiquitination may also regulate the activity of the endocytic machinery itself since Eps15, a protein involved in endocytosis, was found to be monoubiquitinated upon stimulation of cells with EGF [210]. More evidence for a role of monoubiquitination in regulating cellular functions, came from reports on the budding of retroviruses from the plasma membrane [211]. Ubiquitin is implicated in a late step in budding because treatment of cells with proteasome inhibitors reduces the budding-rates by approximately 3-fold. It is thought that proteasome inhibitors deplete the intracellular pool of free ubiquitin and it was shown that overexpression of ubiquitin in inhibitor treated cells can substantially overcome the budding defect [212, 213]. The retroviral Gag protein is the primary structural protein of the virion, it assembles at the plasma membrane of the host cell and is the only viral protein strictly required for virus budding. Within Gag, a small sequence called the late assembly (L)-domain, is essential for budding and particle release. The L-domains contain a P(T/S)AP or PPxY proline rich sequence which can act as an interaction motif with ubiquitin ligases [214]. A small percentage (2 - 5%) of the Gag protein was found to be monoubiquitinated [215] and ubiquitination is dependent on the L-domain [216]. The budding defect, as induced with proteasome inhibitors, is partially restored by the in-frame fusion of ubiquitin to a Gag protein, but a co-requirement for the L-domain remains, suggesting that this domain plays additional roles besides ubiquitination of Gag [212]. Whether the ubiquitination of Gag serves a role in budding or that this modification is simply a reporter for the nearby presence of a ubiquitin ligase, whose real function is to ubiquitinate a cellular protein at the budding site, must be established. Recent data suggest that ubiquitination of Gag itself is not required for viral replication [217].

Numerous plasma membrane proteins undergo ubiquitination, which is in many cases triggered by ligand binding. They are transported, via the endocytic pathway, to the lysosome/vacuole where they are degraded. Mechanisms involved in these processes are poorly understood and the involvement of the ubiquitin system is implicated both at the cell surface in mediating internalization and in the sorting endosome for targeting membrane proteins to the lysosome [reviewed in 106, 171]. Many questions remain: How does ligand binding trigger ubiquitination of membrane cell surface receptor proteins? What are the targeting signals that are identified by the ubiquitin ligases? How does ubiquitination lead to routing of membrane proteins to the lysosome/vacuole? Is the proteasome involved in the targeting? Is monoubiquitination sufficient in all cases or is chain formation via K63 of ubiquitin involved? Which proteins of the cellular

machinery recognize the ubiquitin-modified target proteins and is ubiquitination of the target protein required?

In 1986 the first plasma membrane proteins were found to be ubiquitinated when microsequencing of those proteins revealed two amino-terminal sequences. One of them corresponded to the sequence of ubiquitin [218, 219]. In 1994, Kölling and Hollenberg provided the first evidence in yeast that ubiquitination might be linked to endocytosis when they showed that ubiquitinated ABC-transporter Ste6p accumulated at the cell surface in the mutant yeast strain *end4*Δ [220]. Hicke and Riezmann showed in 1996 that ubiquitination of the yeast G protein-coupled α- factor receptor in fact marked this plasma membrane protein for degradation in the vacuole [221]. Additional evidence for a role of ubiquitination in endocytosis accumulated since that time, but information regarding the targeting signals that specify recognition by the ubiquitination machinery remains limited. In addition, information is scarce concerning the effector proteins that are involved. I will now summarize the available information as much as is known for the individual proteins studied.

ATP-binding cassette (ABC) transporter, Ste6

Ste6p is required for secretion of the mating pheromone a-factor and is mainly associated with internal membranes. In endocytosis mutants, it accumulates at the cell surface in the ubiquitinated form [220], but Ste6 ubiquitination is not connected to proteasomal degradation since its half-life is not altered in proteasome mutant yeast strains. Ste6p is strongly stabilized in a *pep4* mutant, which inhibits vacuolar degradation. A linker region, which connects the two homologous halves of the Ste6, contains a signal (DAKTI) that mediates its ubiquitination and its fast turnover [222]. Recent experiments showed, that Ste6 accumulates at the vacuolar membrane in a yeast mutant defective in the deubiquitinating enzyme Doa4. Expression of additional ubiquitin suppressed the defect of uptake into the lumen of the vacuole, indicating that this defect is the result of reduced ubiquitination. These data suggest that ubiquitination is required for Ste6p sorting into the vacuole [223].

α-factor receptor, Ste2p

Ste2p is a G-protein coupled receptor that spans the membrane seven times, with an extracellular N-terminus and a 134 amino acid cytoplasmic tail. Upon binding α-factor, a 13 amino acid pheromone, the receptor becomes hyperphosphorylated and activates a signal transduction pathway. Ligand binding results in ubiquitination of the receptor and is essential for endocytosis of the ligand-receptor complex as well as its subsequent degradation in the vacuole. Ubiquitin-dependent receptor internalization requires the presence of lysine residues in the receptor cytoplasmic tail and the action of Ubc4 and Ubc5 ubiquitin conjugating enzymes [221]. Receptor phosphorylation, controlled by the serine residues within the SINNDAKSS internalization sequence, promotes internalization and positively regulates ubiquitination [224]. Overexpression of a mutant ubiquitin

in which all lysines were changed to arginines did not affect Ste2p internalization, indicating that monoubiquitination on a single lysine residue is sufficient for internalization [205]. In frame fusions of plasma membrane proteins with ubiquitin were used to show that ubiquitin itself carries the information necessary to promote internalization and that receptor tail sequences are not required [204]. It is suggested that the three-dimensional structure of ubiquitin carries the internalization signal that can be appended to a plasma membrane protein to trigger down-regulation.

a-factor receptor, Ste3p

Like Ste2p, Ste3p is a G-protein coupled receptor and member of the seven transmembrane segment family. Ste3p is subject to two modes of endocytosis, a ligand dependent and a constitutive, ligand independent, endocytosis mode. The constitutive endocytosis is rapid, and as a result, Ste3p is short-lived with a half-life of about 20 min. Its degradation is dependent on vacuolar proteases. Both modes of endocytosis are associated with ubiquitination, and Ste3p ubiquitination increases in mutant yeast, defective in endocytic uptake [225]. Ste3p ubiquitination is impaired in cells that lack the Ubc4 and Ubc5 ubiquitin-conjugating enzymes and that have been depleted of ubiquitin. A large proline/glutamic acid/serine/threonine rich (PEST)-like sequence in the C-terminal domain of Ste3p directs its ubiquitination, internalization and vacuolar degradation [226]. The lysines that provide the attachment site for ubiquitin are located within this PEST-like sequence. Ste3p-ubiquitin in frame fusions that have the ubiquitin moiety fused to the receptor in place of the PEST signal, do undergo rapid endocytosis and vacuolar degradation [208]. Monoubiquitination is sufficient for Ste3p internalization, but multiubiquitination increases the rate of uptake.

General amino acid permease, Gap1p

Gap1p is tightly regulated according to the nitrogen source present in the culture medium. Its activity is maximal in cells using proline or urea as the sole nitrogen source. Upon addition of ammonium ions, synthesis of Gap1p is reduced and the cell surface located permease is internalized and degraded in the vacuole. Ammonium-induced inactivation of Gap1p is accompanied by increased conversion of Gap1p to ubiquitinated forms and requires the Npi1/Rsp5 ubiquitin-ligase [227]. Recently, it was shown that in cells grown on a relatively rich nitrogen source, Gap1p is directed from the Golgi complex to the vacuolar pathway and degraded without ever reaching the cell surface. Two accessory factors, Bul1p and Bul2p, form a complex with Rsp5p and specify the polyubiquitination of Gap1p which is required as a signal for the sorting of Gap1p from the Golgi-complex to the vacuole [154]. By overexpression of single lysine mutant ubiquitin, it was shown that Gap1p is ubiquitinated at two acceptor sites and that ammonium triggers formation of K63-linked polyubiquitin chains. When polyubiquitination through K63 is blocked, the permease still undergoes ammonium-induced down-regulation but to a lesser extent [207]. The Rsp5 C-terminal catalytic HECT-domain, con-

tains the active site Cys, that is required for ubiquitination and subsequent endocytosis of Gap1p. Deletion of the C2 domain of Rsp5 impairs the internalization, without affecting ubiquitination of the permease, suggesting that this domain is important for endocytosis of ubiquitinated Gap1p [228].

Uracil permease, Fur4p

The *FUR4* encoded *S. cerevisiae* uracil permease, is a multispanning plasma membrane protein that has a constitutive turnover which is accelerated under several stress conditions, such as nutrient starvation, heat stress or the inhibition of protein synthesis. Both basal and stress-stimulated degradation are triggered by its ubiquitination, depending on the Npi1/Rsp5p ubiquitin-protein ligase [229]. Like in Gap1p, deletion of the Rsp5p C2-domain does not impair Fur4p ubiquitination but does affect Fur4p internalization [230]. Permease ubiquitination and endocytosis are defective in mutant yeast lacking the Doa4 isopeptidase, a phenotype which can be complemented by the overexpression of ubiquitin. Overproduction of ubiquitin in wild-type cells does not modify the rate of Fur4p internalization or degradation. Using the *ΔDoa4* cells it was shown that overexpression of ubiquitin mutated at K63 did not restore permease ubiquitination. Monoubiquitination is sufficient to induce permease endocytosis, but K63-linked ubiquitin chains stimulate the process [194]. Phosphorylation of the permease at the plasma membrane facilitates its ubiquitination and internalization. The uracil permease is phosphorylated at several serine residues within a N-terminal PEST sequence [231]. Lysine residues adjacent to the PEST sequence are the target sites for ubiquitination; mutation of these lysines to arginines permits phosphorylation, but abolishes internalization of the permease [232]. Fur4p accumulates in the vacuole of *pep4* cells, defective in vacuolar degradation, as an entire protein. This observation indicates that the protein does not undergo proteolytic processing on its way from the plasma membrane to the vacuole and suggests that the proteasome is not involved in proteolysis of the protein [229].

The yeast ZRT1 zinc transporter is inactivated by internalization and degradation in the vacuole due to a raise in the extracellular zinc level. ZRT1 is ubiquitinated upon zinc treatment, and ubiquitination and endocytosis depend on the presence of the Rsp5p ubiquitin-ligase and the Ubc4 and Ubc5 ubiquitin conjugating enzymes. Mutation of Lys-195 in ZRT1 blocks both ubiquitination and endocytosis [233]. The essential step in the down-regulation of the galactose transporter Gal2p is its ubiquitination through the Ubc1p-Ubc4p-Ubc5p triad of ubiquitin-conjugating enzymes and the Npi1/Rsp5 ubiquitin-ligase. Gal2p is stabilized in mutant cells that lack the ubiquitin-hydrolase Npi2p/Doa4p, and the mutant phenotype can be rescued by over-expression of ubiquitin. Over-expression of mutated ubiquitin, which is unable to form polyubiquitin chains, indicated that monoubiquitination of Gal2p is sufficient to signal its internalization [206, 234]. In yeast, ubiquitination of plasma membrane proteins was also detected in the

multidrug transporter Pdr5p [235], the maltose permease [203, 236], and the tryptophan permease Tat2 [237]. In many cases, monoubiquitination appears to be sufficient for endocytosis, and in some cases polyubiquitination via lysine 63 of ubiquitin can stimulate the process. Although it is thought that the ubiquitin moiety itself carries the information necessary for internalization, the endocytic machinery that recognizes ubiquitin is not yet identified. In all the reported cases, genetic evidence suggests that the proteasome plays no role in the ubiquitin-mediated endocytosis in yeast.

The yeast Rsp5p is an HECT-domain ubiquitin ligase that is involved in the endocytosis of most, if not all, of the plasma membrane proteins. At this time however, it is not known how the ligase recognizes its substrate. Rsp5p is a multidomain protein containing an N-terminal C2 domain, three WW-domains, and a C-terminal catalytic HECT-domain. The WW-domain is a small (~40 residues) protein/protein interaction module, which usually binds proline-rich sequences. The plasma membrane proteins which are regulated by Rsp5p lack apparent PY-motifs, but carry acidic sequences, and/or phosphorylation-based sequences that may be important, directly or indirectly, for their recognition by Rsp5p [reviewed in: 130]. Using green fluorescent protein (GFP) fusions to Rsp5p and immunogold electron microscopy, Rsp5p was found distributed at multiple sites within the endocytic pathway, suggesting multiple functions for this ligase at early and late stages of endocytosis. Both the C2-domain and an active HECT-domain are critical for proper localization, although Rsp5p can associate with cellular membranes in the absence of its C2-domain [230, 238, 239]. An active ubiquitin conjugation machinery, including Rsp5p, is required for the constitutive endocytosis of a Ste2p-ubiquitin chimera and for fluid-phase endocytosis. Therefore, in addition to modification of the target protein with ubiquitin, Rsp5p-dependent ubiquitination of, possibly, a component of the endocytic machinery is required for internalization [240].

While Rsp5p plays an important role in the endocytosis of many *S. cerevisiae* plasma membrane proteins, in mammalian cells, a role for Nedd4 (neuronal precursor cell expressed developmentally downregulated, [241]), which is a member of the family of HECT-domain E3 enzymes, has only been shown for the epithelial sodium channel (ENaC). Family members carry an N-terminal Ca^{2+}-dependent lipid/protein binding (C2) domain, two to four WW- domains and a C-terminal catalytic HECT-domain. Nedd4 regulates ENaC, by binding of its WW-domains to specific PY-motifs of the channel. Recently, a novel mouse Nedd4 protein was identified, mNedd4-2, that contains four WW-domains but lacks the C2-domain, and which inhibits ENaC activity when co-expressed in *Xenopus* oocytes. The classical mouse Nedd4, mNedd4-1, which contains a C2-domain and only three WW-domains, was not able to bind and control the activity of ENaC [242]. The number of WW-domains in the Nedd4/Rsp5 family varies between two and four, suggesting that they can form multiple interactions and complexes with proteins simultaneously.

Epithelial Na^+ channel (ENaC)

The amiloride-sensitive ENaC plays an essential role in the reabsorption of fluid across epithelia of the kidney, colon, lung, and ducts of exocrine glands. It facilitates the entry of Na^+ across apical membranes, a process driven by the basolateral Na/K - ATPase [243]. Precise regulation of the channel is essential, and a number of human diseases have been linked to malfunction or mutations in ENaC, including Liddle's syndrome. Liddle's syndrome is a hereditary form of arterial hypertension characterized by an abnormal increase in ENaC activity due to increased retention and opening of the channel at the cell surface [244]. ENaC is composed of a combination of three partially homologous subunits, α, β, and γ, assembled in a tetrameric α2βγ structure at the plasma membrane [245]. The C-terminal region of every subunit contains several conserved proline rich sequences, including the PY-motif (xPPxY; P = proline, Y = tyrosine, x = any amino acid) [246]. These PY-motifs serve as binding sites for the WW-domains of Nedd4, and mutation of the PY-motifs that causes Liddle's syndrome also abrogates binding to this E3 ubiquitin ligase [247]. In accord with its ability to associate with Nedd4, ENaC was found to be regulated by ubiquitination, which takes place primarily on a cluster of lysine residues at the N-terminus of the γ (but also on the α) subunit. Replacing these lysine with arginines results in impaired channel ubiquitination and an increase in channel activity, due to an increased number of ENaC molecules at the cell surface [248]. ENaC is a short-lived protein with a t½ of approximately 1 h for its total cellular pool and also with a short half-life for the cell surface protein. Using several lysosomal and proteasomal inhibitors, it was shown that the properly assembled α2βγENaC is likely targeted for lysosomal degradation, whereas individually expressed ENaC chains are heavily ubiquitinated and rapidly degraded by the proteasome [132]. However, a study in native, sodium-transporting epithelial cells (*Xenopus* distal nephron derived A6 cells), showed that channel function increased when the proteasome was blocked by proteasome inhibitors. ENaC activity was not affected in these cells by lysosomal inhibitors. It was concluded that endogenous functioning ENaC channel proteins in the cell membrane are not degraded by the lysosomes, but by the proteasome, and it was suggested that protein expression levels in transfected cells may change the pathways by which protein turnover is regulated [249]. The proof that Nedd4 is a suppressor of ENaC came when it was demonstrated that overexpression of the E3 leads to an inhibition of channel activity by reducing the number of channels at the cell surface. This effect is not seen upon overexpression of a catalytically inactive Nedd4 [250].

The cardiac voltage-gated Na^+-channel is a PY-motif-containing protein that is also negatively regulated by Nedd4. Therefore, Nedd4 regulation of cell surface proteins possessing PY-motifs, may represent a more general mechanism [251]. Interestingly, a role for ubiquitin ligase recruitment in retrovirus release has recently been suggested [216]. The late assembly domains (L-domain) in retroviruses contain polyproline motifs which match the PPxY core consensus of WW-domain ligands. It was found that the

engagement of the ubiquitin-conjugation machinery by L-domains plays a crucial role in releasing a diverse group of enveloped viruses. In addition, the use of proteasome inhibitors revealed that they can inhibit virus release, probably by reducing the level of free ubiquitin [213, 252]. Plasma membrane proteins could also indirectly interact with Nedd4 via adaptor proteins. For example, the insulin-like growth factor receptor (IGF-1R) binds to Grb10, which in turn binds to Nedd4. Indeed, IGF-1R, but not Grb10, was shown to be ubiquitinated, raising the possibility that association with Grb10 may allow Nedd4 to access the IGF1-R [253, 254].

In addition to the regulation of ubiquitin-mediated endocytosis of plasma membrane proteins by Nedd4 or Rsp5p, several other mammalian cell surface proteins are regulated by ubiquitination, most likely excluding Nedd4 involvement. For several receptor tyrosine kinases (RTKs), such as the epidermal growth factor (EGF)-receptor [255], the Neu oncogene [256], the platelet derived growth factor (PDGF)-receptor [257], and the colony stimulating factor (CSF)-1 receptor [258], the c-Cbl adaptor protein acts as a negative regulator. Recently, c-Cbl was identified as an ubiquitin-ligase whose RING-finger recruits the ubiquitin-conjugating E2 enzyme UbcH7 [140, 259]. c-Cbl exerts its inhibitory effect by binding via its variant SH2 domain to tyrosine phosphorylated RTKs, inducing receptor ubiquitination and endocytosis, thus leading to the removal of the activated receptor from the cell surface and termination of ligand-induced signaling. The site of action of c-Cbl remains controversial.It has been suggested that c-Cbl may act in endosomal sorting [255] but also at the cell surface prior to internalization [258]. The insulin receptor represents an example of a receptor tyrosine kinase that indirectly associates with c-Cbl. The adapter protein APS, containing a PH and SH2 domain, was shown to mediate the recruitment of c-Cbl to the insulin receptor, leading to ubiquitination of the insulin receptor [260]. Ubiquitination of the TCRζ chain is also promoted by c-Cbl and involves an indirect interaction of the E3-ligase with its substrate. In this case, the tyrosine kinase Zap-70, which binds both TCRζ and c-Cbl, functions as an adaptor protein. c-Cbl acts as a negative regulator of T-cell development and activation, possibly by down-regulating the TCR/CD3 complex [261].

Epidermal growth factor receptor (EGF-receptor)

The EGF-receptor is ubiquitinated in response to EGF, but not when a mutant kinase-negative EGF-receptor is used or when clathrin-mediated endocytosis is inhibited by K^+-depletion [108]. If endocytosis is inhibited by overexpressing the GTPase-deficient mutant K44A form of dynamin, the EGF-receptor is transiently localized to coated pits without internalization of EGF-EGF-receptor complexes. Polyubiquitination of the EGF-receptor occurs as efficiently in endocytosis deficient cells as in cells performing normal endocytosis, indicating that EGF-receptor ubiquitination occurs at the plasma membrane prior to endocytosis [262]. It is also proposed that EGF-receptor polyubiquitination occurs in endosomes [255]. The adaptor protein c-Cbl plays an essen-

tial role in the ligand-induced ubiquitination of the EGF-receptor by a mechanism that involves an interaction of its RING finger domain with the ubiquitin conjugating enzyme UbcH7 [139, 141]. Overexpression of c-Cbl enhances down-regulation of activated EGF-receptors from the cell surface and the c-Cbl tyrosine kinase binding domain (TKB) is essential for this activity [263]. Ligand binding to the EGF-receptor results in autophosphorylation on Y-1045, which leads to recruitment of c-Cbl via its SH2-domain and phosphorylation of c-Cbl on Y-371. In turn, this phosphorylation leads to EGF-receptor ubiquitination and degradation [259]. The role of c-Cbl in endocytosis and intracellular sorting is controversial however, since other studies demonstrated that EGF-receptor lysosomal targeting does not require kinase activity [264, 265], and a truncated EGF-receptor at residue 1022 is internalized and degraded at a rate indistinguishable from the full-length receptor [266]. How ubiquitination targets incoming receptors to the lysosomes is unknown as it is also not known whether both lysosomes and proteasomes are involved in the degradation of the cytoplasmic and exoplasmic domains. Proteasome inhibitors interfered with the c-Cbl induced down-regulation of the EGF-receptor, which allowed receptor recycling to occur and suggested a possible role for the proteasome in EGF-receptor down-regulation [255].

Platelet-derived growth factor receptor (PDGF-receptor)

Two types of receptors for PDGF, α and β receptors, were identified, and both belong to the receptor tyrosine kinase subfamily III [219]. The PDGF-β receptor undergoes ligand-induced polyubiquitination, a process that plays a negative regulatory role in its mitogenic signaling, by promoting the efficient degradation of the ligand-activated receptor [109, 267]. Ligand-induced degradation of the PDGF-β receptor is inhibited in the presence of proteasome inhibitors, suggesting a role for the proteasome in the degradation process [268]. c-Cbl enhances the ligand-induced ubiquitination and subsequent degradation of both PDGF-α receptor and PDGF-β receptor and can negatively regulate PDGF-receptor-dependent biological responses, a function that requires the conserved tyrosine kinase binding domain of c-Cbl [257, 269].

Colony stimulating factor-1 receptor (CSF-1 receptor)

Colony stimulating factor-1 (CSF-1) regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells. The effects of CSF-1 on these target cells are mediated by the CSF-1 receptor tyrosine kinase. Addition of CSF-1 to macrophages stimulates the rapid, transient, tyrosine phosphorylation of the CSF-1 receptor, and of c-Cbl which is recruited to the plasma membrane. At the membrane, both the CSF-1 receptor and c-Cbl are polyubiquitinated [270]. The polyubiquitinated CSF-1 receptor is targeted for degradation in the lysosome, while c-Cbl is deubiquitinated and returns to the cytoplasm without degradation [271]. Thus, c-Cbl negatively regulates the macrophage proliferative response to CSF-1, which is associated with the polyubiquitination of CSF-1 receptor and a subsequent increase in the CSF-1 receptor endocytic rate

[258]. The role of polyubiquitinated c-Cbl in this case remains unclear.

These examples show that for several receptor tyrosine kinases, c-Cbl is the E3 ubiquitin-ligase that mediates poly-ubiquitination and targets these receptors for degradation in the lysosome. But a key question remains: Does ubiquitination only play a role in trafficking of membrane proteins to the lysosome or is this modification also involved in receptor down-regulation by targeting the substrate to the 26S proteasome? Unlike in yeast, no genetic approach is available in mammalian cells as a tool to study a possible role of the proteasome. Therefore, proteasome inhibitors are used to investigate the involvement of this multisubunit protease. The most frequently used inhibitors are the peptide aldehydes, such as MG-132. These agents are substrate analogues and potent transition state inhibitors, primarily interfering with the chymotrypsin-like activity of the proteasome. They are highly potent, and cell viability and growth is generally not affected for 10-20 h. The peptide aldehydes also inhibit certain lysosomal cysteine proteases and the calpains. Therefore, it is important to show that similar biological effects occur with other, more specific, proteasome inhibitors (e.g. lactacystin), and that selective inhibitors of lysosomal function do not produce similar effects. Lactacystin and its derivative *clasto*-lactacystin-β-lactone, are natural products structurally different from the peptide aldehydes. They inhibit proteasome function by acting as a pseudosubstrate that becomes linked covalently to the hydroxyl groups on the active site threonine of the proteasome β-subunits. Although lactacystin shows high specificity for the proteasome, it can also inhibit cathepsin A. [272, 273]. For the PDGF-receptor [268], the Met receptor [112], rhodopsin [274], Her2/neu (c-ErbB-2) [275], the neurothrophin receptor TrkB [276], the EGF-receptor [255] and the non-tyrosine kinase erythropoietin (Epo) receptor [277], interleukin-9 receptor [278], interleukin-2 receptor [279], and GHR [280], studies with proteasome inhibitors demonstrated the involvement of the ubiquitin-proteasome pathway in the degradation of these receptors. For the T-cell receptor (TCR) and the *c-kit* receptor, which both are ubiquitinated in response to stimulation, it was shown that their degradation is insensitive to proteasome inhibitors [171]. The degradation of many of these receptors is also inhibited by lysosomal inhibitors, like chloroquine, ammonium chloride and leupeptin indicating that proteasomes and lysosomes may cooperate in the degradation process. The following examples summarize the available data, obtained with the use of proteasome inhibitors, for some of these receptors.

Met-receptor

The Met tyrosine kinase is a high affinity receptor for hepatocyte growth factor/scatter factor (HGF/SF). Met degradation is induced by ligand binding and this degradation can be blocked with the proteasome inhibitor lactacystin. The Met receptor is polyubiquitinated in response to ligand and these polyubiquitinated molecules are stabilized in the presence of lactacystin. Degradation and polyubiquitination of Met is dependent

on its receptor tyrosine kinase activity [112]. A recent study revealed that Met degradation also requires endocytosis and trafficking to late endosomal compartments and that Met degradation is inhibited using a vacuolar-ATPase inhibitor, thus preventing endosomal acidification. In addition, Hammond and co-workers showed that proteasome inhibitors interfere with the internalization of the Met-receptor, while internalization of transferrin and the EGF-receptor was not affected. Therefore, proteasome activity may be indirectly required for Met degradation [281].

Erythropoietin receptor (EPO-receptor)

The EPO-receptor is a member of the cytokine receptor family and expressed almost exclusively on erythroid precursor cells. The EPO-receptor, which controls the development of red blood cells, has no intrinsic kinase activity, but binds JAK2 to elicit its signals. The EPO-receptor has a short half-life of about ~1 h, and demonstrates low cell surface expression; most of the receptors are retained within the ER and degraded [282, 283]. The EPO-receptor is internalized from the cell surface after ligand binding and degraded in lysosomes [284]. Activation of JAK2 and phosphorylation of the EPO-receptor are not required for EPO-induced down-regulation [285]. In the presence of proteasome inhibitors, both the EPO-receptor and STAT5 remain phosphorylated, indicating the involvement of the proteasome in the down-regulation of the EPO-receptor activation signals [277]. Verdier and co-workers showed that this effect is due to the continuous replenishment of the cell surface pool of EPO-receptor by newly synthesized receptors in the presence of proteasome inhibitors. Internalization and degradation of EPO-EPO-receptor complexes is not modified by proteasome inhibitors [286]. Incubation of a Chinese hamster cell line, containing a thermo-labile ubiquitin activating enzyme E1, at the non-permissive temperature, did not interfere with EPO-receptor internalization [285].

Interleukin-2 receptor (IL-2 receptor)

The interleukin-2 (IL-2) receptor is comprised of three subunits: an α-chain, which functions solely in IL-2 binding, and β- and common γ (γc)-chains that both function in ligand binding and signal-transduction [287]. Signal-transduction results from IL-2 induced trimerization of the α, β and γc subunits, which brings the cytoplasmic tails of the β and γc subunits in close proximity, allowing receptor phosphorylation by the associated tyrosine kinases JAK1 and JAK3. IL-2/IL-2 receptor interaction results in receptor-mediated endocytosis of the receptor-ligand complex, which is primarily dependent upon the cytoplasmic tail of γc . In T- lymphocytes, IL-2 is rapidly internalized, ultimately leading to lysosomal degradation. The IL2-receptor α chain recycles to the plasma membrane from an early endosomal compartment, whereas the β- and γc-subunits traffic to the lysosome, where they are degraded [288]. Proteasome inhibitors completely prevented the lysosomal degradation of IL-2 without interfering with the internalization of the IL-2/IL-2 receptor complex. Proteasome function is also necessary for

degradation of IL-2, internalized by an IL-2 receptor comprised of cytoplasmic tailless β- or γc-subunits. This suggests that the target protein for the proteasome is independent of either the cytoplasmic tail of the IL-2R β- or γc-subunits and their associated signaling components [279].

Growth hormone receptor (GHR)

By using a Chinese hamster lung cell line (ts20) with a temperature sensitive mutation in the ubiquitin-activating enzyme, E1, that expresses the wild-type GHR, it was shown that endocytosis and degradation of the GHR are dependent on a functional ubiquitin-conjugation system. Polyubiquitination of the GHR is enhanced upon binding of GH at the permissive temperature and completely inhibited at the non-permissive temperature [113]. Examination of truncated receptors, the endocytosis deficient receptor mutant F327A, and conditions that inhibit clathrin-mediated endocytosis, shows that GHR ubiquitination and ligand-induced internalization are coupled events [99]. Surprisingly, internalization of the truncated receptor GHR (399K271-362R), that is not ubiquitinated because all cytoplasmic lysine residues are replaced with arginines, is still dependent on a functional ubiquitin-conjugation system. A specific domain in the GHR cytoplasmic tail regulates receptor endocytosis via the ubiquitin-conjugation system. This domain, the Ubiquitin-dependent Endocytosis motif (UbE- motif), consists of the amino acid sequence DSWVEFIELD [114]. In addition to the UbE-motif, the cytoplasmic tail of the GHR contains a di-leucine motif at position 347-348. Upon truncation of the receptor at amino acid residue 349, this di-leucine motif is activated and mediates ubiquitin-system independent internalization of the GHR. Di-leucine mediated GHR internalization requires functional clathrin-coated pits and results in GHR transport to the lysosome. The full-length GHR internalizes independent of the di-leucine motif [289].

In summary, modification of proteins by addition of ubiquitin can alter protein levels, protein function and protein localization. Regulation of the cell surface availability of receptors, permeases and transporters can be controlled by the ubiquitin-proteasome pathway in many different ways. (1) The quality control system of the ER and the ER-associated degradation regulate the amount of, for example, the EPO-receptor transported to the cell surface. (2) Depending on the quality of the external nitrogen source, Gap1p is directed, in a Rsp5p dependent mechanism, from the Golgi complex to the vacuolar sorting pathway and degraded without reaching the cell surface. (3) At the cell surface, internalization of many plasma membrane proteins is regulated by ubiquitination mediated by, for example, Rsp5p, Nedd4 or c-Cbl. (4) In the sorting endosome, ubiquitination regulates sorting of the EGF-receptor and Ste6p into the multivesicular bodies pathway. Additional regulation can be achieved by a variety of deubiquitinating enzymes, such as Dot4p that can inhibit the down-regulation of plasma membrane proteins by preventing their endocytosis [290].

Regulation of GHR availability by the ubiquitin-proteasome pathway may have important clinical consequences. GHR deficiency has a phenotype of severe growth retardation and is associated with the GH insensitivity syndrome (GHIS). Inherited GHIS is rare and due to mutations of the GHR gene (Laron syndrome), but acquired or secondary GHIS is common and found in most sick patients particularly those with malnutrition. The catabolic conditions that have been associated with acquired GHIS include nutritional deficiency, diabetes mellitus, hepatic insufficiency, and renal failure and are accompanied with reduced GHBP levels, indicating decreased GHR levels [82, 291]. Alternatively, the ubiquitin-proteasome pathway is believed to be the main system for degradation of muscle protein in catabolic condition, induced by starvation [292], sepsis [293], metabolic acidosis [294], denervation atrophy [295], burns [296] and diabetes mellitus [297]. These two independent clinical observations in catabolic disease indicate that up-regulation of the ubiquitin-proteasome pathway in severe illness is accompanied by down-regulation of the GHR, resulting in GHR deficiency and acquired GHIS.

OUTLINE OF THIS THESIS

Ubiquitination is a post-translational modification that can regulate protein levels, protein localization and receptor signaling in a variety of different ways. In this thesis, we focused on the involvement of the ubiquitin-proteasome pathway in the regulation of the cell surface expression of the GHR, and thus the GHR availability for signaling.

We found that GHR ubiquitination occurs at the cell surface and coincides with its recruitment to clathrin-coated membrane domains, indicating that the ubiquitin-conjugation system and the classical endocytic machinery may cooperate to regulate the time-span of the GHR at the cell surface (Chapter II). GHR internalization requires proteasome action in addition to an active ubiquitin-conjugation system. In the presence of specific proteasome inhibitors, internalization of the full-length GHR is inhibited, while a truncated GHR can enter the cell independent of proteasome action (Chapter III). Internalized receptors are recycled back to the plasma membrane or degraded in lysosomes. With the use of specific proteasome inhibitors, we could inhibit lysosomal degradation of internalized ligands that remain bound to their receptor. This observation suggests a role for the ubiquitin-proteasome pathway in intracellular sorting of selected membrane proteins to lysosomes (Chapter IV). Finally, we defined the processes that determine GHR degradation, and thus GHR availability for GH at the cell surface. With the use of a monovalent GH antagonist, B2036, in which the GHR binding site 2 is mutated, we could quantify both the internalization and the shedding of the "unoccupied" receptor (Chapter V). By comparing internalization with shedding, we show that internalization followed by lysosomal degradation is the major pathway for receptor degradation. In addition, evidence is provided that the ubiquitin-proteasome pathway controls the half-life of the cell surface expressed GHR by controlling its constitutive internalization.

REFERENCES

1 Evans, H. M. and Long, J. A. (1921) Anat. Rec. **21**, 61-63.

2 Smith, P. E. (1930) Am. J. Anat. **45**, 205-273.

3 Li, C. H. and Papkoff, H. (1956) Science **124**, 1293-1294.

4 Li, C. H. and Evans, H. M. (1944) Science **99**, 183-184.

5 Beck, J., McGarry, E., Dyrenfurth, R. and Venning, E. (1957) Science **125**, 884-885.

6 Knobil, E. and Greep, R. (1959) Recent Prog. Horm. Res. **15**, 1-68.

7 Salmon, W. D. and Daughaday, W. H. (1957) J. Lab. Clin. Med. **49**, 825-836.

8 Elders, M. J., Wingfield, B., McNatt, M., *et al*. (1975) Ann. Clin. Lab. Sci. **5**, 440-451.

9 D'Ercole, A., Applewhite, G. and Underwood, L. (1980) Dev. Biol. **75**, 315-328.

10 Green, H., Morikawa, M. and Nixon, T. (1985) Differentiation **29**, 195-198.

11 Le Roith, D., Bondy, C., Yakar, S., *et al.* (2001) Endocr. Rev. **22**, 53-74.

12 Clark, R., Mortensen, D. and Carlsson, L. (1995) Endocrine **3**, 297-304.

13 Miller, L. and Eberhardt, N. (1983) Endocr. Rev. **4**, 97-129.

14 Wallace, A. and Ferguson, K. (1961) J. Endocrinol. **23**, 285-290.

15 Waters, M. J., Shang, C. A., Behncken, S., *et al*. (1999) Clin. Exp. Pharm. Phys., **26**, 760-764.

16 Kostyo, J. L. (1968) Ann. NY Acad. Sci. **148**, 389-407.

17 Miller, J. D., Tannenbaum, G., Colle, E., *et al*. (1982) J. Clin. Endocrinol. Metab. **55**, 989-994.

18 Jansson, J., Albertsson Wikland, K., Eden, S., *et al*. (1982) Acta Physiol. Scand. **114**, 261-265.

19 Casanueva, F. (1992) Endocrinol. Metab. Clin. North Amer. **21**, 483-517.

20 Baumann, G., Stolar, M., Amburn, K., *et al.* (1986) J. Clin. Endocrinol. Metabol. **62**, 134-141.

21 Herington, A. C., Ymer, S. I. and Stevenson, J. (1986) J. Clin. Invest. **77**, 1817-1823.

22 Leung, D. W., Spencer, S. A., Cachianes, G., *et al*. (1987) Nature **330**, 537-544.

23 Baumann, G., Amburn, K. and Shaw, M. (1988) Endocrinology **122**, 976-984.

24 Veldhuis, J. D., Johnson, M., Faunt, L., *et al*. (1993) J. Clin. Invest. **91**, 629-641.

25 Barnard, R. and Waters, M. J. (1997) J. Endocrinol. **153**, 1-14.

26 Posner, B., Kelly, P. A., Shiu, R. and Friesen, H. (1974) Endocrinology **95**, 521-531.

27 Waters, M. J. (1999) Handbook of Physiology, **vol. 5**, Oxford University Press, NY, 397-444.

28 Asakawa, K., Hedo, J., Gorden, P., *et al*. (1988) Acta Endocrinol.Copenh. **119**, 517-524.

29 Waters, M. J. and Friesen, H. (1979) J. Biol. Chem. **254**, 6815-6825.

30 Fuh, G., Mulkerrin, M. G., Bass, S., *et al*. (1990) J. Biol. Chem. **265**, 3111-3115.

31 Zhang, Y., Jiang, J., Kopchick, J. J. and Frank, S. J. (1999) J. Biol. Chem. **274**, 33072-33084.

32 Amit, T., Baram, G., Dastot, F., *et al* (1999) Endocrinology **140**, 266-272.

33 Baumgartner, J. W., Wells, C. A., Chen, C. M., *et al*. (1994) J. Biol. Chem. **269**, 29094-29101.

34 Firmbach-Kraft, I., Byers, M., Shows, R., *et al*. (1990) Oncogene **5**, 1329-1336.

35 Argetsinger, L. S., Campbell, G. S., Yang, X. N., *et al*. (1993) Cell **74**, 237-244.

36 Cosman, D., Lyman, S., D., Idzerda, R., *et al.* (1990) Trends Biochem.Sci. **15**, 265-270.

37 Godowski, P. J., Leung, D. W., *et al*. (1989) Proc. Natl. Acad. Sci. USA **86**, 8083-8087.

38 Colosi, P., Wong, K., Leong, S. R. and Wood, W. I. (1993) J. Biol. Chem. **268**, 12617-12623.

39 Wang, Y. D. and Wood, W. I. (1995) Mol. Endocrinol. **9**, 303-311.

40 Billestrup, N., Allevato, G., Norstedt, G., *et al*. (1994) Proc. Soc. Exp Biol. Med. **206**, 205-209.

41 Moldrup, A., Allevato, G., Dyrberg, T., *et al*. (1991) J. Biol. Chem. **266**, 17441-17445.

42 Moller, C., Emtner, M., Arner, P. and Norstedt, G. (1994) Mol. Cell. Endocrinol. **99**, 111-117.

43 Emtner, M., Mathews, L. and Norstedt, G. (1990) Mol. Endocrinol. **4**, 2014-2020.

44 van der Kuur, J. A., Wang, X., Zhang, L., *et al*. (1994) J. Biol. Chem. **269**, 21709-21717.

45 Cunningham, B. C., Ultsch, M., deVos, A. M., *et al*. (1991) Science **254**, 821-825.

46 DeVos, A. M., Ultsch, M. and Kossiakoff, A. A. (1992) Science **255**, 306-312.

47 Wells, J. A., Cunningham, B. C., Fuh, G., *et al*. (1993) Recent. Prog. Horm. Res. **48**, 253-275.

48 Fuh, G., Cunningham, B. C., Fukunaga, R., *et al*. (1992) Science **256**, 1677-1680.

49 Mellado, M., Rodriguez-Frade, J. M., Kremer, L., *et al*. (1997) J. Biol. Chem. **272**, 9189-9196.

50 Rowlinson, S. W., Behncken, S. N., Rowland, J. E., *et al*. (1998) J. Biol. Chem. **273**, 5307-5314.

51 Smit, L. S., *et al* (1999) Handbook of Physiology, **vol. 5**, Oxford University Press, NY, 445-480.

52 Carter-Su, C. and Smit, L. S. (1998) Recent Prog. Horm. Res. **53**, 61-82.

53 Aringer, M., Cheng, A., Nelson, J. W., *et al*. (1999) Life Sciences **64**, 2173-2186.

54 Sotiropoulos, A., Perrot Applanat, M., *et al*. (1994) Endocrinology **135**, 1292-1298.

55 Campbell, G. S., Christian, L. J. and Carter Su, C. (1993) J. Biol. Chem. **268**, 7427-7434.

56 Gronowski, A. M. and Rotwein, P. (1994) J. Biol. Chem. **269**, 7874-7878.

57 Silva, C. M., Weber, M. J. and Thorner, M. O. (1993) Endocrinology **132**, 101-108.

58 Pawson, T. and Schlessinger, J. (1993) Curr. Biol. **3**, 434-442.

59 Darnell, J., Kerr, I. and Stark, G. (1994) Science **264**, 1415-1421.

60 Wojcik, J. and Postelvinay, M. C. (1999) Growth Hormone & IGF Research **9**, 51-55.

61 Winston, L. A. and Hunter, T. (1995) J. Biol. Chem. **270**, 30837-30840.

62 van der Kuur, J. A., Butch, E. R., Waters, S. B., *et al*. (1997) Endocrinology **138**, 4301-4307.

63 Cobb, M. and Goldsmith, E. (1995) J. Biol. Chem. **270**, 14843-14846.

64 Ridderstrale, M., Degerman, E. and Tornqvist, H. (1995) J. Biol. Chem. **270**, 3471-3474.

65 Souza, S. C., Frick, G. P., Yip, R., Lobo, R. B., *et al*. (1994) J. Biol. Chem. **269**, 30085-30088.

66 Argetsinger, L. S., Norstedt, G., Billestrup, N., *et al*. (1996) J. Biol. Chem. **271**, 29415-29421.

67 Laron, Z., Pertzelan, A. and Mannheimer, S. (1966) Isr. J. Med. Sci. **2**, 152-155.

68 Ross, R. J. M. (1999) Growth Horm. IGF Res. **9**, 42-45.

69 Rosenbloom, A. L. (2000) Endocrine **12**, 107-119.

70 Ayling, R. M., Ross, R. J. M., Towner, P., *et al*. (1999) Acta Paediatrica **88**, 168-172.

71 Iida, K., Takahashi, Y., Kaji, H., *et al.* (1999) J. Clin. Endocrinol. Metab. **84**, 1011-1016.
72 Dastot, F., Sobrier, M., *et al*. (1996) Proc. Natl. Acad. Sci. USA **93**, 10723-10728.
73 Ross, R. J., Esposito, N., Shen, X. Y., *et al*. (1997) Mol. Endocrinol. **11**, 265-273.
74 Hackett, R. H., Wang, Y. D., Sweitzer, S., *et al*. (1997) J. Biol. Chem. **272**, 11128-11132.
75 Stofega, M. R., Argetsinger, L. S., Wang, H. Y., *et al*. (2000) J. Biol. Chem. **275**, 28222-28229.
76 Adams, T. E., Hansen, J. A., Starr, R., *et al*. (1998) J. Biol. Chem. **273**, 1285-1297.
77 Tollet-Egnell, P., Floresmorales, A., *et al*. (1999) Endocrinology **140**, 3693-3704.
78 Krebs, D. L. and Hilton, D. J. (2000) J. Cell Sci. **113**, 2813-2819.
79 Strous, G., van Kerkhof, P., Govers, R., *et al*. (1997) J. Biol. Chem. **272**, 40-43.
80 Alves Dos Santos, C. M., van Kerkhof, P., *et al*. (2001) J. Biol. Chem. **276**, 10839-10846.
81 Alves Dos Santos, C. M., ten Broeke, T. and Strous, G. J. (2001) J. Biol. Chem. **276,** *in press.*
82 Amit, T., Youdim, M. B. H. and Hochberg, Z. (2000) J. Clin. Endocrinol. Metab. **85**, 927-932.
83 Ilondo, M., Courtoy, P. J., Geiger, D., *et al*. (1986) Proc. Natl. Acad. Sci. USA **83**, 6460-6464.
84 Saito, Y., Teshima, R., Yamazaki, T., *et al*. (1994) Mol. Cell. Endocrinol. **106**, 67-74.
85 Roupas, P., Rettenmier, C., Look, A. and Herington, A. (1987) Endocrinology **120**, 2158-2165.
86 Murphy, L. J. and Lazarus, L. (1984) Endocrinology **115**, 1625-1632.
87 Gorin, E. and Goodman, H. M. (1985) Endocrinology **116**, 1796-1805.
88 Baumann, G. (1994) J. Endocrinol. **141**, 1-6
89 Kelly, P. A., Ali, S., Rozakis, M., *et al*. (1993) Recent. Prog. Horm. Res. **48**, 123-164.
90 Edens, A., Southard, J. N. and Talamantes, F. (1994) Endocrinology **135**, 2802-2805.
91 Zhou, Y., He, L. and Kopchick, J. J. (1994) Receptor **4**, 223-227.
92 Alele, J., Jiang, J., Goldsmith, J. F., *et al*. (1998) Endocrinology **139**, 1927-1935.
93 Guan, R., Zhang, Y., Jiang, J., Baumann, *et al*. (2001) Endocrinology **142**, 1137-1147.
94 Amit, T., Hochberg, Z., Yogev-Falach, M., *et al*. (2001) J. Endocrinol. **169**, 397-407.
95 Zhang, Y., Jiang, J., Black, R. A., Baumann, G. *et al*. (2000) Endocrinology **141**, 4342-4348.
96 Zhang, Y., Guan, R., Jiang, J., *et al.* (2001) J. Biol. Chem., **276**, 24565-24573.
97 Roupas, P. and Herington, A. (1988) Mol. Cell. Endocrinol. **57**, 93-99.
98 Ilondo, M. M., Smal, J., DeMeyts, P. and Courtoy, P. J. (1991) Endocrinology **128**, 1597-1602.
99 Govers, R., van Kerkhof, P., Schwartz, A. L. and Strous, G. J. (1997) EMBO J. **16**, 4851-4858.
100 Barazzone, P., Lesniak, M. A., Gorden, P., *et al*. (1980) J. Cell Biol. **87**, 360-369.
101 Yamada, K., Lipson, K. E. and Donner, D. B. (1987) Biochemistry **26**, 4438-4443.
102 Roupas, P. and Herington, A. (1989) Mol. Cell. Endocrinol. **61**, 1-12.
103 Lobie, P. E., Barnard, R. and Waters, M. J. (1991) J. Biol. Chem. **266**, 22645-22652.
104 Goldsmith, J., Lee, S., Jiang, J., *et al*. (1997) Amer. J. Physiol- Endocrinol. Met. **36**, E932-E941.
105 Ilondo, M. M., Vanderschueren Lodeweyckx, M., *et al*. (1992) Endocrinology **130**, 2037-2044.

106 Strous, G. J. and Govers, R. (1999) J. Cell Sci. **112**, 1417-1423.

107 Hicke, L. (2001) Nat. Rev. Mol. Cell Biol. **2**, 195-201.

108 Galcheva-Gargova, Z., Theroux, S. J. and Davis, R. J. (1995) Oncogene **11**, 2649-2655.

109 Mori, S., Heldin, C. H. and Claesson-Welsh, L. (1992) J. Biol. Chem. **267**, 6429-6434.

110 Miyazawa, K., Toyama, K., Gotoh, A., *et al*. (1994) Blood **83**, 137-145.

111 Cenciarelli, C., Hou, D., Hsu, K. C., Rellahan, B. L., *et al*. (1992) Science **257**, 795-797.

112 Jeffers, M., Taylor, G. A., Weidner, K. M., *et al*. (1997) Mol. Cell. Biol. **17**, 799-808.

113 Strous, G., van Kerkhof, P., Govers, R., Ciechanover, A. *et al*. (1996) EMBO J. **15**, 3806-3812.

114 Govers, R., ten Broeke, T., van Kerkhof, P., *et al*. (1999) EMBO J. **18**, 28-36.

115 Ciechanover, A., Orian, A. and Schwartz, A. L. (2000) Bioessays **22**, 442-451.

116 Hershko, A. and Ciechanover, A. (1998) Annu. Rev. Biochem. **67**, 425-479.

117 Schwartz, A. L. and Ciechanover, A. (1999) Annu. Rev. Med. **50**, 57-74.

118 Weissman, A. M. (2001) Nat. Rev. Mol. Cell Biol. **2**, 169-178.

119 Pickart, C. M. (2001) Annu. Rev. Biochem. **70**, 503-533.

120 Koegl, M., Hoppe, T., Schlenker, S., *et al.* (1999) Cell **96**, 635-644.

121 Handley Gearhart, P., Stephen, A. G., *et al*. (1994) J. Biol. Chem. **269**, 33171-33178.

122 Finley, D., Ciechanover, A. and Varshavsky, A. (1984) Cell **37**, 43-55.

123 Jentsch, S., Seufert, W., Sommer, T. and Reins, H. A. (1990) Trends Biochem. Sci. **15**, 195-198.

124 Mathias, N., Steussy, C. and Goebl, G. (1998) J. Biol. Chem. **273**, 4040-4045.

125 Sommer, T. and Jentsch, S. (1993) Nature **365**, 176-179.

126 Huang, L., Kinnucan, E., Wang, G. L., *et al*. (1999) Science **286**, 1321-1326.

127 Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000) Cell **102**, 533-539.

128 Huibregtse, J., Scheffner, M., *et al*. (1995) Proc. Natl. Acad. Sci. USA **92**, 2563-2567.

129 Joazeiro, C. and Weissman, A. M. (2000) Cell **102**, 549-552.

130 Rotin, D., Staub, O. and Haguenauer-Tsapis, R. (2000) J. Membrane Biol. **176**, 1-17.

131 Hicke, L. (1999) Trends Cell Biol. **9**, 107-112.

132 Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., *et al*. (1997) EMBO J. **16**, 6325-6336.

133 Joazeiro, C. A. P. and Hunter, T. (2000) Science **289**, 2061-2062.

134 Kamura, T., Koepp, D. M., Conrad, M. N., *et al*. (1999) Science **284**, 657-661.

135 Ohta, T., Michel, J., Schottelius, A. and Xiong, Y. (1999) Mol. Cell **3**, 535-541.

136 Lorick, K., Jensen, J., Fang, S., *et al*. (1999) Proc. Natl. Acad. Sci. USA **96**, 11364-11369.

137 Fang, S. Y., Jensen, J. P., Ludwig, R. L., *et al*. (2000) J. Biol. Chem. **275**, 8945-8951.

138 Honda, R., Tanaka, H. and Yasuda, H. (1997) FEBS Letters **420**, 25-27.

139 Waterman, H., Levkowitz, G., Alroy, I. and Yarden, Y. (1999) J. Biol. Chem. **274**, 22151-22154.

140 Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., *et al*. (1999) Science **286**, 309-312.

141 Yokouchi, M., Kondo, T., Houghton, A., *et al*. (1999) J. Biol. Chem. **274**, 31707-31712.

142 Yang, Y., Fang, S., Jensen, J., Weissman, A. M. and Ashwell, J. (2000) Science **288**, 874-877.

143 Jackson, P. K., Eldridge, A. G., Freed, E., *et al*. (2000) Trends Cell Biol. **10**, 429-439.

144 Deshaies, R. J. (1999) Annu. Rev. Cell Dev. Biol. **15**, 435-467.

145 Winston, J. T., Koepp, D. M., *et al.* (1999) Curr. Biol. **9**, 1180-1182.

146 Zhou, P. B., Bogacki, R., McReynolds, L. and Howley, P. M. (2000) Mol. Cell **6**, 751-756.

147 Zhang, J., Farley, A., Nicholson, S., *et al* (1999) Proc. Natl. Acad. Sci. USA **96**, 2071-2076.

148 Stebbins, C. E., Kaelin, W. G., Jr. and Pavletich, N. P. (1999) Science **284**, 455-461.

149 Iwai, K., Yamanaka, K., Kamura, T., *et al*. (1999) Proc. Natl. Acad. Sci. USA **96**, 12436-12441.

150 Maxwell, P. H. (1999) Nature **399**, 271-275.

151 Glotzer, M., Murray, A. W. and Kirschner, M. W. (1991) Nature **349**, 132-138.

152 Pfleger, C. and Kirschner, M. (2000) Genes Dev. **14**, 655-665.

153 Page, A. and Hieter, P. (1999) Annu. Rev. Biochem. **68**, 583-609.

154 Helliwell, S., Losko, S. and Kaiser, C. (2001) J. Cell Biol. **153**, 649-662.

155 Wilkinson, K. D. (1997) FASEB J. **11**, 1245-1256.

156 Chung, C. H. and Baek, S. H. (1999) Biochem. Biophys. Res. Commun. **266**, 633-640.

157 Huang, Y., Baker, R. T. and Fischer-Vize, J. A. (1995) Science **270**, 1828-1831.

158 Zhu, Y. A., Lambert, K., Corless, C., *et al*. (1997) J. Biol. Chem. **272**, 51-57.

159 Coux, O., Tanaka, K. and Goldberg, A. L. (1996) Annu. Rev. Biochem. **65**, 801-847.

160 Kloetzel, P. M. (2001) Nat. Rev. Mol. Cell Biol. **2**, 179-187.

161 Hilt, W. and Wolf, D. H. (1996) Trends Biochem.Sci. **21**, 96-102.

162 Cardozo, C., Chen, W. E. and Wilk, S. (1996) Arch. Biochem. Biophys. **334**, 113-120.

163 Glickman, M. (1999) Mol. Biol. Reprod. **26**, 21-28.

164 Hoppe, T. and Jentsch, S. (2000) Cell **102**, 577-586.

165 Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. (1994) Cell **78**, 773-785.

166 Orian, A., Whiteside, S., Israel, A., *et al*. (1995) J. Biol. Chem. **270**, 21707-21714.

167 Sears, C., Olesen, J., Rubin, D., Finley, D. *et al*. (1998) J. Biol. Chem. **273**, 1409-1419.

168 Murakami, Y. (1992) Nature **360**, 597-599.

169 Sheaff, R. J., Singer, J. D., Swanger, J., *et al*. (2000) Mol. Cell **5**, 403-410.

170 Plemper, R. K. and Wolf, D. H. (1999) Mol. Biol. Rep. **26**, 125-130.

171 Bonifacino, J. S. and Weissman, A. M. (1998) Annu. Rev. Cell Dev. Biol. **14**, 19-57.

172 Hiller, M. M., Finger, A., Schweiger, M. and Wolf, D. H. (1996) Science **273**, 1725-1728.

173 Biederer, T., Volkwein, C. and Sommer, T. (1996) EMBO J. **15**, 2069-2076.

174 Werner, E., Brodsky, J. and McCracken, A.(1996) Proc. Natl. Acad. Sci. USA **93**, 13797-13801.

175 Hampton, R. Y. and Bhakta, H. (1997) Proc. Natl. Acad. Sci. USA **94**, 12944-12948.

176 Biederer, T., Volkwein, C. and Sommer, T. (1997) Science **278**, 1806-1809.
177 Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998) Mol. Biol. Cell **9**, 209-222.
178 Knop, M., Finger, A., Braun, T., Hellmuth, K. and Wolf, D. H. (1996) EMBO J. **15**, 753-763.
179 Plemper, R., Bordallo, J., Deak, P., *et al*. (1999) J. Cell Sci. **112**, 4123-4134.
180 Deak, P. M. and Wolf, D. H. (2001) J. Biol. Chem. **276**, 10663-10669.
181 Yu, H., Kaung, G., Kobayashi, S. and Kopito, R. R. (1997) J. Biol. Chem. **272**, 20800-20804.
182 Yu, H. and Kopito, R. R. (1999) J. Biol. Chem. **274**, 36852-36858.
183 Jensen, T., Loo, M., Pind, S., Williams, D., *et al*. (1995) Cell **83**, 129-135.
184 Ward, C. L., Omura, S. and Kopito, R. R. (1995) Cell **83**, 121-127.
185 Yang, M., Omura, S., Bonifacino, J. S. and Weissman, A. M. (1998) J. Exp. Med. **187**, 835-846.
186 Tiwari, S. and Weissman, A. M. (2001) J. Biol. Chem. **276**, 16193-16200.
187 Dubiel, W. and Gordon, C. (1999) Curr. Biol. **9**, R554-R557.
188 Pickart, C. M. (2000) Trends Biochem. Sci. **25**, 544-548.
189 Pickart, C. M. (1997) FASEB J. **11**, 1055-1066.
190 Finley, D., Sadis, S., Monia, B. P., *et al*. (1994) Mol. Cell. Biol. **14**, 5501-5509.
191 Spence, J., Sadis, S., Haas, A. L. and Finley, D. (1995) Mol. Cell. Biol. **15**, 1265-1273.
192 Arnason, T. and Ellison, M. J. (1994) Mol. Cell. Biol. **14**, 7876-7883.
193 Fisk, H. A. and Yaffe, M. P. (1999) J. Cell Biol. **145**, 1199-1208.
194 Galan, J. M. and Haguenauer-Tsapis, R. (1997) EMBO J. **16**, 5847-5854.
195 Spence, J. *et al.* (2000) Cell **102**, 67-76.
196 Hofman, R. and Pickart, C. (1999) Cell **96**, 645-653.
197 Deng, L., Wang, C., Spencer, E., Yang, L. Y., *et al*. (2000) Cell **103**, 351-361.
198 West, M. H. and Bonner, W. M. (1980) Nucl. Acids Res. **8**, 4671-4680.
199 West, M. H. P. and Bonner, W. M. (1980) Biochemistry **19**, 3238-3245.
200 Mueller, R. D., Yasuda, H., Hatch, C. L., *et al.* (1985) J. Biol. Chem. **260**, 5147-5153.
201 Robzyk, K., Recht, J. and Osley, M. (2000) Science **287**, 501-504.
202 Pham, A. and Sauer, F. (2000) Science **289**, 2357-2360.
203 Lucero, P., Penalver, E., Vela, L. and Lagunas, R. (2000) J. Bacteriol. **182**, 241-243.
204 Shih, S. C., Sloper-Mould, K. E. and Hicke, L. (2000) EMBO J. **19**, 187-198.
205 Terrell, J., Shih, S., Dunn, R. and Hicke, L. (1998) Mol. Cell **1**, 193-202.
206 Horak, J. and Wolf, D. H. (2001) J. Bacteriol. **183**, 3083-3088.
207 Springael, J. Y., Galan, J. M., Haguenauer-Tsapis, R., *et al*. (1999) J. Cell Sci. **112**, 1375-1383.
208 Roth, A. F. and Davis, N. G. (2000) J. Biol. Chem. **275**, 8143-8153.
209 Nakatsu, F., Sakuma, M., Matsuo, Y., *et al.* (2000) J. Biol. Chem. **275**, 26213-26219.
210 van Delft, S., Govers, R., Strous, G. J., *et al*. (1997) J. Biol. Chem. **272**, 14013-14016.

211 Vogt, V. M. (2000) Proc. Natl. Acad. Sci. USA **97**, 12945-12947.

212 Patnaik, A., Chau, V. and Wills, J. W. (2000) Proc. Natl. Acad. Sci. USA **97**, 13069-13074.

213 Schubert, U., Ott, D., Chertova, E, *et al*. (2000) Proc. Natl. Acad. Sci. USA **97**, 13057-13062.

214 Harty, R., Brown, M., Wang, G., *et al*. (2000) Proc. Natl. Acad. Sci. USA **97**, 13871-13876.

215 Ott, D. E., Coren, L. V., Copeland, T. D., *et al*. (1998) J. Virology **72**, 2962-2968.

216 Strack, B., Calistri, A., Accola, M., *et al*. (2000) Proc. Natl. Acad. Sci. USA **97**, 13063-13068.

217 Ott, D. E., Coren, L. V., Chertova, E. , *et al.* (2000) Virology **278**, 111-121.

218 Siegelman, M., Bond, M. W., Gallatin, W. M., *et al*. (1986) Science **231**, 823-829.

219 Yarden, Y., Escobedo, J. A., Kuang, W. J., *et al*. (1986) Nature **323**, 226-231.

220 Kölling, R. and Hollenberg, C. P. (1994) EMBO J. **13**, 3261-3271.

221 Hicke, L. and Riezman, H. (1996) Cell **84**, 277-287.

222 Kölling, R. and Losko, S. (1997) EMBO J. **16**, 2251-2261.

223 Losko, S., Kopp, F., Kranz, A. and Kölling, R. (2001) Mol. Biol. Cell **12**, 1047-1059.

224 Hicke, L. (1997) FASEB J. **11**, 1215-1226.

225 Roth, A. F. and Davis, N. G. (1996) J. Cell Biol. **134**, 661-674.

226 Roth, A. F., Sullivan, D. M. and Davis, N. G. (1998) J. Cell Biol. **142**, 949-961.

227 Springael, J. Y. and Andre, B. (1998) Mol. Biol. Cell **9**, 1253-1263.

228 Springael, J. Y., Decraene, J. O. *et al*. (1999) Biochem. Biophys. Res. Commun. **257**, 561-566.

229 Galan, J. M., Moreau, V., Andre, B., *et al*. (1996) J. Biol. Chem. **271**, 10946-10952.

230 Wang, G., McCaffery, J. M., Wendland, B., *et al*. (2001) Mol. Cell. Biol. **21**, 3564-3575.

231 Marchal, C., Haguenauer-Tsapis, R. and Urbangrimal, D. (1998) Mol. Cell. Biol. **18**, 314-321.

232 Marchal, C., Haguenauer-Tsapis, R., *et al*. (2000) J. Biol. Chem. **275**, 23608-23614.

233 Gitan, R. S. and Eide, D. J. (2000) Biochem. J **346**, 329-336.

234 Horak, J. and Wolf, D. H. (1997) J. Bacteriol. **179**, 1541-1549.

235 Egner, R. and Kuchler, K. (1996) FEBS Letters **378**, 177-181.

236 Medintz, I., Wang, X., Hradek, T. and Michels, C. A. (2000) Biochemistry **39**, 4518- 4526.

237 Beck, T., Schmidt, A. and Hall, M. N. (1999) J. Cell Biol. **146**, 1227-1237.

238 Dunn, R. and Hicke, L. (2001) Mol. Biol. Cell **12**, 421-435.

239 Gajewska, B., Kaminska, J., Jesionowska, A., *et al*. (2001) Genetics **157**, 91-101.

240 Dunn, R. and Hicke, L. (2001) J. Biol. Chem. **276**, 25974-25981.

241 Kumar, S., Tomooka, Y., *et al.* (1992) Biochem. Biophys. Res. Commun. **185**, 1155-1161

242 Kamynina, E., Debonneville, C., *et al*. (2001) FASEB J. **15**, 204-214.

243 Garty, H. and Palmer, L. (1997) Physiol. Rev. **77**, 359-396.

244 Abriel, H., Loffing, J., Rebhun, J. F., *et al.* (1999) J. Clin. Invest. **103**, 667-673.

245 Firsov, D., Gautschi, I., Merillat, A., Rossier, B. and Schild, L. (1998) EMBO J. **17**, 344-352.

246 Schild, L., Lu, Y., Gautschi, I., Schneeberger, E., *et al*. (1996) EMBO J. **15**, 2381-2387.
247 Staub, O., Dho, S., Henry, P. C., Correa, J., *et al.* (1996) EMBO J. **15**, 2371-2380.
248 Staub, O., Abriel, H., Plant, P., Ishikawa, T., *et al*. (2000) Kidney Int. **57**, 809-815.
249 Malik, B., Schlanger, L., Al-Khalili, O., *et al*. (2001) J. Biol. Chem. **276**, 12903-12910.
250 Harvey, K. F. and Kumar, S. (1999) Trends Cell Biol. **9**, 166-169.
251 Abriel, H., Kamynina, E., Horisberger, J. D. and Staub, O. (2000) FEBS Letters **466**, 377-380.
252 Ikeda, M., Ikeda, A., Longan, L. C. and Longnecker, R. (2000) Virology **268**, 178-191.
253 Morrione, A., Plant, P., Valentinis, B., Staub, O., *et al.* (1999) J. Biol. Chem. **274**, 24094-24099.
254 Sepp-Lorenzino, L., Ma, Z., Lebwohl,E., *et al*. (1995) J. Biol. Chem. **270**, 16580-16587.
255 Levkowitz, G., Waterman, H., Zamir, E., *et al*. (1998) Genes Dev. **12**, 3663-3674.
256 Levkowitz, G., Oved, S., Klapper, L. N., *et al*. (2000) J. Biol. Chem. **275**, 35532-35539.
257 Miyake, S., Lupher, M., Druker, B., *et al*. (1998) Proc. Natl. Acad. Sci. USA **95**, 7927-7932.
258 Lee, P. S., Wang, Y., Dominguez, M. G., *et al*. (1999) EMBO J. **18**, 3616-3628.
259 Levkowitz, G., Waterman, H., Ettenberg, S. A., *et al*. (1999) Mol. Cell **4**, 1029-1040.
260 Ahmed, Z., Smith, B. J. and Pillay, T. S. (2000) Febs Letters **475**, 31-34.
261 Wang, H., Altman, Y., Fang, D., *et al* (2001) J. Biol. Chem. **276**, 26004-26011.
262 Stang, E., Johannessen, L. E., *et al*. (2000) J. Biol. Chem. **275**, 13940-13947.
263 Lill, N. L., Douillard, P., Awwad, R. A., *et al*. (2000) J. Biol. Chem. **275**, 367-377.
264 Kil, S. J., Hobert, M. and Carlin, C. (1999) J. Biol. Chem. **274**, 3141-3150.
265 Opresko, L. K., Chang, C. P., Will, B. H., *et al*. (1995) J. Biol. Chem. **270**, 4325-4333.
266 Wiley, H. S., Herbst, J. J., Walsh, B. J., *et al*. (1991) J. Biol. Chem. **266**, 11083-11094.
267 Mori, S., Heldin, C. H. and Claesson-Welsh, L. (1993) J. Biol. Chem. **268**, 577-583.
268 Mori, S., Kanaki, H., Tanaka, K., *et al*. (1995) Biochem. Biophys. Res. Commun. **217**, 224-229.
269 Miyake, S., Mullane-Robinson, K. P., Lill, N. L., *et al*. (1999) J. Biol. Chem. **274**, 16619-16628.
270 Wang, Y., Yeung, Y. G., Langdon, W. Y. and Stanley, E. R. (1996) J. Biol. Chem. **271**, 17-20.
271 Wang, Y., Yeung, Y. G. and Stanley, E. R. (1999) J. Cell Biochem. **72**, 119-134.
272 Lee, D. H. and Goldberg, A. L. (1998) Trends Cell Biol. **8**, 397-403.
273 Rock, K. L., Gramm, C., Rothstein, L., *et al*. (1994) Cell **78**, 761-771.
274 Obin, M. S., Jahngenhodge, J., *et al*. (1996) J. Biol. Chem. **271**, 14473-14484.
275 Mimnaugh, E. G., Chavany, C. and Neckers, L. (1996) J. Biol. Chem. **271**, 22796-22801.
276 Sommerfeld, M. T., Schweigreiter, R., *et al*. (2000) J. Biol. Chem. **275**, 8982-8990.
277 Verdier, F., Chretien, S., Muller, O., *et al*. (1998) J. Biol. Chem. **273**, 28185-28190.
278 Yen, C., Yang, Y., Ruscetti, S., *et al*. (2000) J. Immunol. **165**, 6372-6380.
279 Yu, A. and Malek, T. (2001) J. Biol. Chem. **276**, 381-385.
280 van Kerkhof, P., Govers, R., Alves Dos Santos, C., *et al*. (2000) J. Biol. Chem. **275**, 1575-1580.

281 Hammond, D., Urbé, S., Van de Woude, G. and Clague, M. (2001) Oncogene, **20**, 2761-2770.

282 Levin, I., Cohen, J., Supino-Rosin, L., *et al*. (1998) FEBS Letters **427**, 164-170.

283 Supino-Rosin, L., Yoshimura, A., *et al*. (1999) Eur. J. Biochem. **263**, 410-419.

284 Sawyer, S., Krantz, S. and Goldwasser, E. (1987) J. Biol. Chem. **262**, 5554-5562.

285 Beckman, D. L., Lin, L. L., Quinones, M. E. and Longmore, G. D. (1999) Blood **94**, 2667-2675.

286 Verdier, F., Walrafen, P., Hubert, N., *et al.* (2000) J. Biol. Chem. **275**, 18375-18381.

287 Yu, A., Olosz, F., Choi, C. Y. and Malek, T. R. (2000) J. Immunol. **165**, 2556-2562.

288 Hemar, A., Subtil, A., Lieb, M., Morelon, E. and Hellio, R. (1995) J. Cell Biol. **129**, 55-64.

289 Govers, R., van Kerkhof, P., Schwartz, A. L. *et al*. (1998) J. Biol. Chem. **273**, 16426-16433.

290 Kahana, A. (2001) Biochem. Biophys. Res. Commun. **282**, 916-920.

291 Ross, R. J. and Chew, S. (1995) Eur. J. Endocrinol. **132**, 655-660.

292 Wing, S., Haas, A. and Goldberg, A. L. (1995) Biochem. J. **307**, 639-645.

293 Tiao, G., Fagan, J., Samuels, N., James, J., *et al.* (1994) J. Clin. Invest. **94**, 2255-2264.

294 Mitch, W. E., Medina, R., Greiber, S., *et al*. (1994) J. Clin. Invest. **93**, 2127-2133.

295 Medina, R., Wing, S. S. and Goldberg, A. L. (1995) Biochem. J. **307**, 631-637.

296 Fang, C. H., Tiao, G., James, J., *et al.* (1995) J. Am. Coll. Surg. **180**, 161-170.

297 Price, S. R., Bailey, J., Wang, X., *et al*. (1996) J. Clin. Invest. **98**, 1703-1708.

Chapter II

GROWTH HORMONE RECEPTOR UBIQUITINATION COINCIDES WITH RECRUITMENT TO CLATHRIN-COATED MEMBRANE DOMAINS

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ABSTRACT

Endocytosis of the growth hormone receptor (GHR) depends on a functional ubiquitin conjugation system. A 10-amino acid residue motif within the GHR cytosolic tail (the ubiquitin-dependent endocytosis motif) is involved in both GHR ubiquitination and endocytosis. As shown previously, ubiquitination of the receptor itself is not required. In this paper ubiquitination of the GHR was used as a tool to address the question of at which stage the ubiquitin conjugation system acts in the process of GHR endocytosis. If potassium depletion was used to interfere with early stages of coated pit formation, both GHR endocytosis and ubiquitination were inhibited. Treatment of cells with methyl-β-cyclodextrin inhibited endocytosis at the stage of coated vesicle formation. Growth hormone addition to methyl-β-cyclodextrin-treated cells resulted in an accumulation of ubiquitinated GHR at the cell surface. Using immunoelectron microscopy, the GHR was localized in flattened clathrin-coated membranes. In addition, when clathrin-mediated endocytosis was inhibited in HeLa cells expressing a temperature-sensitive dynamin mutant, ubiquitinated GHR accumulated at the cell surface. Together, these data show that the GHR is ubiquitinated at the plasma membrane, before endocytosis occurs, and indicate that the resident time of the GHR at the cell surface is regulated by the ubiquitin conjugation system together with the endocytic machinery.

Abbreviations used:

GHR, growth hormone receptor; GH, growth hormone; MβCD, methyl-β-cyclodextrin; mAb, monoclonal antibody; HA, hemagglutinin; MEM, minimal essential medium; dyn^{TS}, temperature-sensitive mutant of dynamin; wtdyn, wild-type dynamin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EGF, epidermal growth factor.

INTRODUCTION

Clathrin-mediated endocytosis involves the formation of clathrin-coated vesicles from coated pits at the plasma membrane. Recruitment of membrane proteins into clathrin-coated pits is mediated by specific amino acid sequences within their cytoplasmic domain (for review, see Refs. 1, 2). The best-defined coated pit localization signals are the tyrosine-based motifs NPXY as described for, e.g. the low-density lipoprotein receptor (3), and YXXϕ (where ϕ is an amino acid with a bulky hydrophobic group) found in, e.g. the transferrin receptor (4). Alternatively, internalization of the insulin and β2-adrenergic receptor is mediated by a dileucine-containing motif (5, 6). Many receptors, including the low-density lipoprotein receptor and the transferrin receptor, are clustered in coated pits and internalized constitutively, independent of ligand occupancy. The heterotetrameric adaptor complex AP-2 binds directly to the tyrosine-based motif and nucleates assembly of clathrin triskelions onto the plasma membrane (for review, see Ref. 7). Invagination of the plasma membrane results in the formation of constricted coated pits, followed by the dynamin-dependent detachment of coated vesicles from the plasma membrane. More complex situations exist when plasma membrane proteins enter cells on stimuli such as hormone binding or specific signal transduction events. In this case the internalization signal is only recognized on ligand binding, or the stimulus induces the addition of a signal, which results in the subsequent recruitment of the receptor into the coated pit. The agonist-induced phosphorylation of the β2-adrenergic receptor resulting in the binding of β-arrestin, a specialized adaptor that binds to clathrin, is an example of a protein modification that regulates internalization (8). Recently, it was shown that the attachment of ubiquitin moieties is involved in the internalization of several plasma membrane proteins (for review, see Refs. 9, 10). In mammalian cells, the ubiquitin conjugation system regulates the endocytosis of the epithelial sodium channel (11) and the growth hormone receptor (GHR; Ref. 12).

The GHR was initially found to be ubiquitinated on amino acid sequencing of the receptor from rabbit liver (13). The ubiquitin conjugation system is involved in GHR internalization and degradation (12, 14). In particular, a 10-amino acid motif within the GHR cytosolic tail (the ubiquitin-dependent endocytosis motif, DSWVEFIELD) is involved in both receptor ubiquitination and endocytosis (15). We have recently shown that the proteasome is also involved in GHR internalization (16). Ligand-induced internalization of the GHR is blocked in the presence of specific proteasomal inhibitors such as carbobenzoxy-L-leucyl-L-leucyl-L-leucinal and β-lactone, the more membrane-permeable analogue of lactacystin. Disruption of clathrin-mediated endocytosis by cellular potassium depletion (17), hypertonic medium treatment (18), or cellular cytosol acidification (19) inhibits internalization and ubiquitination of the GHR (14). Although ubiquitination of the GHR itself is not required for endocytosis (15), GHR internalization requires the activity of the ubiquitin conjugation system, which acts together with the

endocytic machinery in targeting the receptor into the coated pit.

In this study the question was addressed of at which stage the ubiquitin conjugation system acts in the process of GHR endocytosis. GHR ubiquitination was taken as a biochemical marker for the location of the activity of the ubiquitin conjugation system. Conditions in which coated pit formation was prevented were compared with conditions that allowed coated pit formation but prevented coated vesicle formation.

EXPERIMENTAL PROCEDURES

Materials and Antibodies

Antibody mAb5 recognizing the lumenal part of the GHR was from AGEN Inc. (Parsippany, NJ). Polyclonal antibodies against amino acid residues 271-318 of the cytosolic tail of the GHR (anti-T; Ref. 16) and against human growth hormone (GH) were raised in rabbits. Antiserum specific for protein-ubiquitin conjugates was a generous gift from Dr. A. Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). Antibody 4G10 (anti-pY), recognizing phosphotyrosine residues, was obtained from Upstate Biotechnologies Inc. (Lake Placid, NY). Anti-biotin was from Rockland (Gilbertsville, PA); monoclonal anti-clathrin was from Transduction Laboratories (Lexington, NY); anti-mouse IgG was from Nordic Immunological Laboratories (Tilburg, The Netherlands); and monoclonal anti-hemagglutinin (HA) antibody 12CA5 was from Babco (Richmond, CA). Human GH was a kind gift from Lilly; methyl-β-cyclodextrin (MβCD) was from Sigma; LipofectAMINE was from Life Technologies, Inc.; and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal was from Calbiochem.

Plasmids, Cell Culture, and Transfection

Wild-type rabbit GHR cDNA was cloned into the cytomegalovirus-Neo expression plasmid pcDNA3.1 (Invitrogen BV/Novex) and used for transient transfections. The internalization-deficient mutant GHR(F327A) was constructed by site-directed mutagenesis and cloned into pcDNA3.1 as described (20). The Chinese hamster cell line ts20, stably transfected with a pCB6 construct containing the rabbit GHR cDNA sequence, was used in this study (12). Cells were grown at 30°C in MEMα supplemented with 10% fetal calf serum, 4.5 g/l glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.45 mg/ml geneticin. For experiments cells were grown on 35- or 60-mm dishes in the absence of geneticin to ~75% confluence, and 10 mM sodium butyrate was added overnight to increase GHR expression (12). tTA-HeLa cell lines stably transfected with the HA-tagged temperature-sensitive mutant of dynamin (dyn^{TS}) or wild-type dynamin (wtdyn) were kindly provided by Dr. S. Schmid (The Scripps Research Institute, La Jolla, CA). Dyn^{TS} carries a mutation of the glycine at position 273 to aspartic acid. Cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.4 mg/ml geneticin, 2 μg/ml tetracycline, and 200 ng/ml puromycin. For transfection experiments subconfluent cultures of tTA-HeLa cells were washed with phosphate-buffered saline (PBS), detached with trypsin-EDTA, plated on 60-mm dishes, and incubated at the permissive temperature of 32°C in the absence of tetracycline for 24 h. Cells were 30-40% confluent when transfected with 1 μg cDNA/dish, using LipofectAMINE according to the manufacturer's protocol. After 24 h, cells were washed with medium free of tetracycline. Cells were used for experiments 48 h after transfection and 72 h after removal of tetracycline.

GH Binding and Internalization

^{125}I-Human GH was prepared using chloramine T (12). For internalization studies, cells were grown in 12-well cluster plates, washed with MEMα supplemented with 20 mM Hepes, pH 7.4, and 0.1% bovine serum albumin (BSA), and incubated in a water bath. ^{125}I-GH (8 nM) was bound on ice for 2 h, and the cells were washed free of unbound GH and incubated for 0-30 min at 30°C. Membrane-associated GH was removed by acid wash (0.15 M NaCl, 50 mM glycine, 0.1% BSA, pH 2.5) on ice. Internalized GH was determined by measuring the radioactivity after solubilization of the acid-treated cells in 1 M NaOH using an LKB gamma counter.

Metabolic Labeling

Cells were grown in 60-mm dishes and incubated in methionine- and cysteine-free MEM. Then 3.7 MBq/ml [^{35}S]methionine (Tran^{35}S Label,, 40 TBq/mmol; ICN, Costa Mesa, CA) was added, and the incubation was continued at 30°C in a CO_2 incubator. The radioactivity was replaced with medium containing 100 μM unlabeled methionine, 0.1% BSA, and 8 nM GH and chased for 0-60 min. Cells were lysed, and samples were immunoprecipitated (see below). Radioactivity was determined using a Storm imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image Quant software, version 4.2a.

Cell Lysis, Immunoprecipitation, and Western Blotting

Immunoprecipitations were performed as described previously (12). For GHR immunoprecipitations, cells were lysed on ice in 0.3 ml of lysis mix containing 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μM carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, and 1mM phenylmethylsulfonyl fluoride in PBS. In ubiquitin immunoprecipitation experiments the cells were lysed in 0.3 ml boiling lysis buffer containing 1% SDS, 1 mM EDTA, 50 mM NaF, and 1 mM Na_3VO_4. The lysate was heated for 5 min at 100°C, after which the DNA was sheared using a 25-G needle. Immunoprecipitation of the supernatant was carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, and 0.5% BSA in PBS plus various inhibitors. For anti-GH immunoprecipitations of GH-GHR complexes, the cells were lysed on ice in a lysis mix containing 1% Triton X-100, 150 mM NaCl, 10% glycerol, 50 mM Tris-HCl, pH 8.0, 10 mM N-ethylmaleimide, and various inhibitors. The immunoprecipitation was carried out in the same buffer. The lysates were incubated with the indicated antibodies for 2 h on ice, and immune complexes were isolated using protein A-agarose beads (Repligen Co., Cambridge, MA). The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. Immune complexes were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as described (14). For detection the enhanced chemiluminescence system (Roche Molecular Biochemicals) was used. To reprobe blots, the membranes were incubated for 1 h at room temperature in 0.15 M NaCl, 50 mM glycine, pH 2.5, buffer. The efficiency of the stripping procedure was checked and was found to remove >95% of the signal.

Potassium Depletion

Cells were subjected to potassium depletion as described previously (14, 18). All incubation steps were performed at 30°C. Cells were washed twice with isotonic, potassium-free buffer A, (0.14 M NaCl, 20 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 g/l glucose, and 0.1% BSA, pH 7.4) and subjected to a hypotonic shock for 5 min in buffer A diluted 1:1 with H_2O. After incubation in buffer A for 30 min, GH was added for an additional 30 min. Parallel cultures, which had also been hypotonically shocked, were incubated in buffer A supplemented with 10 mM KCl.

Light Microscopy

Fluorescently labeled GH (Cy3-GH) was prepared as described before (16). Cells grown on coverslips were incubated in MEMα supplemented with 20 mM Hepes, pH 7.4, and 0.1% BSA. Cy3-GH (0,8 μg/ml) was added, and the incubation was continued. Cells were washed with PBS to remove unbound label and fixed for 2 h in 3% paraformaldehyde in PBS. After fixation, the cells were embedded in Mowiol, and confocal laser scanning microscopy was performed using a Leica TCS 4D system.

Immunogold Electron Microscopy

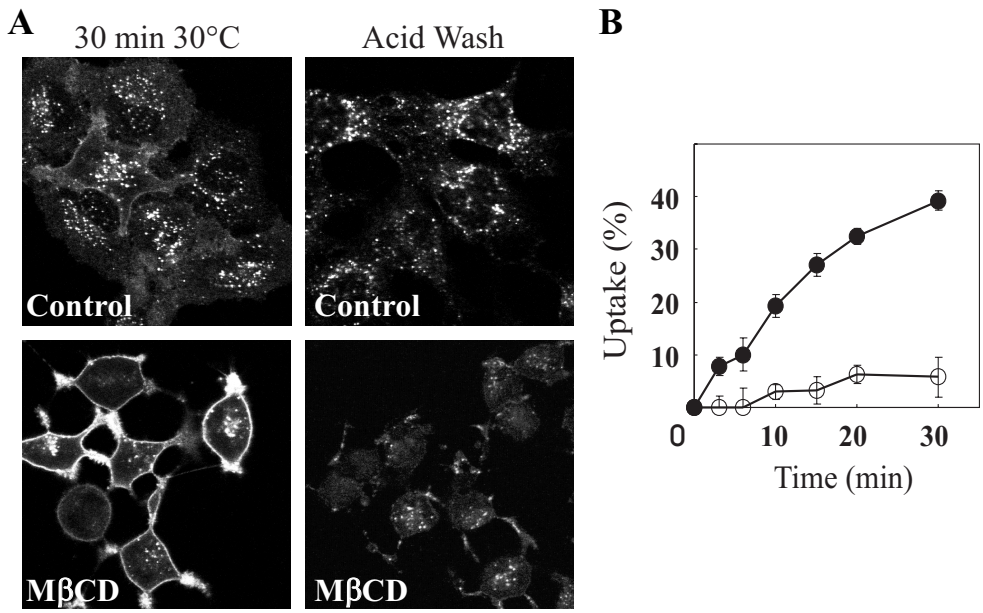
Cells were incubated in MEMα plus 0.1% BSA and 8 nM biotinylated GH (21) in a CO_2 incubator. Before fixation and processing for immunoelectron microscopy, cells were washed three times with MEMα and 0.1% BSA. The cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min on ice followed by 3 h at room temperature. Further processing for ultrathin cryosectioning and labeling according to the protein A-gold method was done as described previously (22). To pick up ultrathin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used (23). A rabbit polyclonal antibody against biotin was directly visualized by protein A-gold. A mouse monoclonal antibody against clathrin was first incubated with rabbit anti-mouse IgG, to provide binding sides for protein A, which binds poorly to mouse antibodies. The effect of MβCD on clathrin-coated pit morphology was quantified by determining the number of clathrin-coated structures at the plasma membrane. MβCD-treated and control cells were labeled for clathrin, and the plasma membrane of 50 cell profiles with visible nucleus was screened for clathrin-coated structures, which were subdivided into four categories: 1) flat or slightly invaginated coated pits, 2) invaginated coated pits with a wide opening, 3) invaginated coated pits with a constricted neck, and 4) vesicles in close vicinity to the plasma membrane, which were not connected to the plasma membrane in the plane of the section. The frequency of each category was expressed as a percentage of the total number of clathrin-coated structures at the plasma membrane.

RESULTS

MβCD Inhibits the Endocytosis of the GHR

The GHR enters the cell via clathrin-coated pits (14, 24, 25). Previously we used methods that deplete the cytosol of free clathrin triskelions and interfere with the early stages of coated pit formation (18, 26). These methods inhibited GHR internalization and abolished GHR ubiquitination (14), suggesting that GHR ubiquitination occurs during or after coated pit formation. Acute cholesterol depletion of the plasma membrane using MβCD inhibits clathrin-coated pit budding. This method reduces the internalization of the transferrin receptor by >85% (27). Cholesterol depletion results in the formation of shallow coated pits, indicating that cholesterol is essential for clathrin coated vesicle formation (28). To monitor GH uptake, GHR-expressing ts20 cells were incubated for 30 min in the presence of Cy3-labeled GH, which resulted in the presence of Cy3-GH in endosomal and lysosomal compartments (Fig. 1A, Control). As expected, the Cy3-GH was protected against acid treatment, confirming that the label is in intracellular structures (Fig. 1A, Acid Wash). When cells were preincubated in the presence of MβCD, virtually no Cy3-GH entered the cells (Fig. 1A, MβCD), and the majority of label could be removed on acid treatment (Fig. 1A, Acid Wash). Neither uptake nor

Fig. 1. Effect of MβCD on GH internalization.

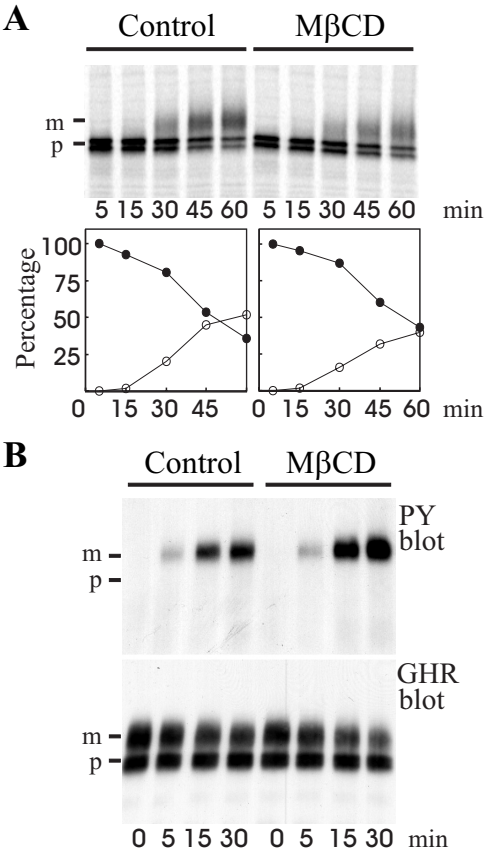


A, GHR-expressing ts20 cells were incubated for 30 min at 30°C in the absence (Control) or presence of 10 mM MβCD and incubated with Cy3-GH for 30 min. Cells were fixed before (30 min, 30°C) or after acid wash (Acid Wash). Cy3-GH was visualized by confocal microscopy.
B, GHR-expressing ts20 cells were incubated for 30 min at 30°C in the absence (Control) or presence of 10 mM MβCD and put on ice for 2 h with ^{125}I-GH. Unbound label was removed, and the cells were incubated at 30°C in the absence or presence of MβCD as indicated. Background label was determined in the presence of excess unlabeled GH and subtracted. The amounts of ^{125}I-GH internalized are plotted as a percentage of the cell-associated radioactivity at the start of the incubation. Each point represents the mean value of two experiments performed in duplicate ± S.D. ●, control; ○, MβCD.

binding was observed when excess unlabeled ligand was added together with Cy3-GH or when untransfected cells were used (data not shown).

To confirm and quantify the effect of MβCD on endocytosis, uptake of ^{125}I-GH was measured in a time course experiment. As seen in Fig. 1B, MβCD inhibited the internalization of GH efficiently. There was no effect of MβCD on the total binding of ^{125}I-GH to the cells (data not shown). Two control experiments were performed to ascertain that MβCD treatment did not affect other relevant cellular processes. The effect of MβCD on GHR biosynthesis was measured using pulse-chase labeling with [^{35}S]methionine (Fig. 2A). The receptor was synthesized as a 110-kDa glycoprotein precursor (double band; Fig. 2A, p) and on "complex glycosylation" in the Golgi complex converted to a 130-kDa mature species (Fig. 2A, m). Quantification of the radioactivity showed that the GHR signal in the MβCD cells is ~85-90% of the control cells, indicating a slight inhibition of protein synthesis. Conversion of precursor to mature recep-

Fig. 2. Effect of MβCD on the biosynthesis and the tyrosine-phosphorylation of the GHR.

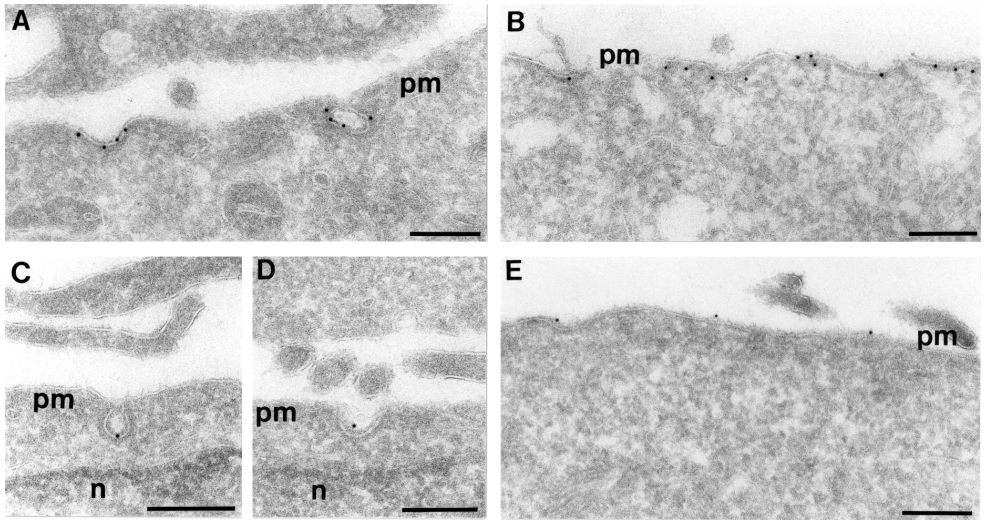


A, GHR-expressing ts20 cells were incubated for 30 min at 30°C in methionine-free medium in the absence (Control) or presence of 10 mM MβCD. [^{35}S]Methionine was added, and the incubation was continued for 10 min. Cells were chased in MEMα supplemented with 0.1% BSA, 100 μM methionine, and 8 nM GH in the absence or presence of MβCD for the periods indicated. *Upper panel*, GHR was immunoprecipitated using anti-T; p, precursor GHR (110 kDa.); m, mature GHR (130 kDa.). *Lower panel*, radioactivity was quantified and expressed as a percentage of the radioactivity incorporated in the precursor GHR. ●, precursor GHR; ○, mature GHR.

B, GHR-expressing ts20 cells were incubated at 30°C in the absence (Control) or presence of 10 mM MβCD. All dishes were incubated for 60 min in total; GH was present during the last 5, 15, or 30 min. *Upper panel*, cells were lysed, and the GHR was immunoprecipitated with anti-T and immunoblotted using anti-pY. *Lower panel*, the same blot was reprobed using anti-GHR (mAb5). p, precursor GHR (110 kDa.); m, mature GHR (130 kDa.).

tor was detectable after 30 min of chase both in control and in MβCD-treated cells, indicating that transport to the Golgi compartment was not affected by the cholesterol depletion. To examine the effect of MβCD on GHR phosphorylation, a second control experiment was performed. Allevato and colleagues (29) showed that a mutated GHR, deficient in internalization, was capable of stimulating transcription of the serine protease inhibitor 2.1 promoter, and we showed that the GHR cytosolic tail is tyrosine-phosphorylated, while internalization is inhibited (14, 20). From these studies it was concluded that GHR phosphorylation is independent of GHR endocytosis. MβCD-pretreated cells were incubated for various periods with GH. As seen in Fig. 2B, phosphorylation became detectable after 5 min of incubation, reaching a maximum after 15 min for both control and MβCD-treated cells. The blot was reprobed with anti-GHR to show that comparable amounts of receptor were loaded in each lane. From these results we conclude that MβCD treatment has no effect on GH-induced phosphorylation of the GHR, indicating that activation of the tyrosine kinase (Janus kinase 2) and receptor dimerization can take place in the presence of MβCD.

Fig. 3. MβCD does not effect accessibility of clathrin-coated pits for GHR.



Ultrathin cryosections show plasma membrane (pm) regions of GHR-expressing ts-20 cells incubated 30 min without (A, C, and D) or with (B and E) 10 mM MβCD before addition of biotinylated GH. Sections were labeled for clathrin (A and B) or biotinylated GH (C-E). A, in control cells, clathrin (10-nm gold) was associated with invaginated clathrin-coated pits. The dense cytosolic coating is characteristic of the presence of clathrin. B, in MβCD-treated cells, clathrin (10-nm gold) assembled in longer stretches along the plasma membrane, and only flattened invaginations were formed. C and D, biotinylated GH (10-nm gold) had access to deeply invaginated clathrin-coated pits in control cells, as well as to the flattened clathrin-coated pits in MβCD-treated cells (E). n, nucleus. Scale bars, 200 nm.

MβCD Does Not Affect Accessibility of Clathrin-coated Pits for GHR

Previously, it was shown that MβCD does not interfere with the association of clathrin to the plasma membrane but has an inhibitory effect on the invagination and fission of clathrin-coated pits (28). For the present study it was important to determine whether the GHR enters these clathrin-coated areas at the plasma membrane of MβCD-treated cells. Immunogold labeling of clathrin in GHR-expressing ts20 cells revealed that the majority of plasma membrane-associated clathrin was present on deeply invaginated pits and coated vesicles in close vicinity to the plasma membrane (Fig. 3A and Table I). GH was regularly found in the deeply invaginated clathrin-coated pits and vesicles (Fig. 3, C and D), as well as in later compartments of the endocytic pathway (data not shown). In MβCD-treated cells, the total number of clathrin-coated structures at the plasma membrane was increased twice compared with that in control cells. In contrast to control cells, >85% of the clathrin-containing structures were flattened coated pits (Fig. 3B). Deeply invaginated coated pits and coated vesicles were rarely seen (Table I). GH accumulated at the plasma membrane, where it regularly but not exclusively occurred in the flattened clathrin-coated pits (Fig. 3E). These findings suggest

Table I. Percentage of clathrin-coated structures at the plasma membrane.

	1	2	3	4
control	27	30	28	15
+MβCD	87	8	3	2

In MβCD-treated and control cells, clathrin-coated structures at the plasma membrane of 50 cell profiles were counted and categorized into the four indicated stages (see "Experimental Procedures" for details). The occurrence of a specific stage is expressed as percentage of the total number of clathrin-coated structures per condition. In MβCD-treated and control cells 310 and 145 clathrin-coated structures were counted, respectively.

that the GH-GHR complex in MβCD-treated cells indeed enters the clathrin-coated areas of the plasma membrane.

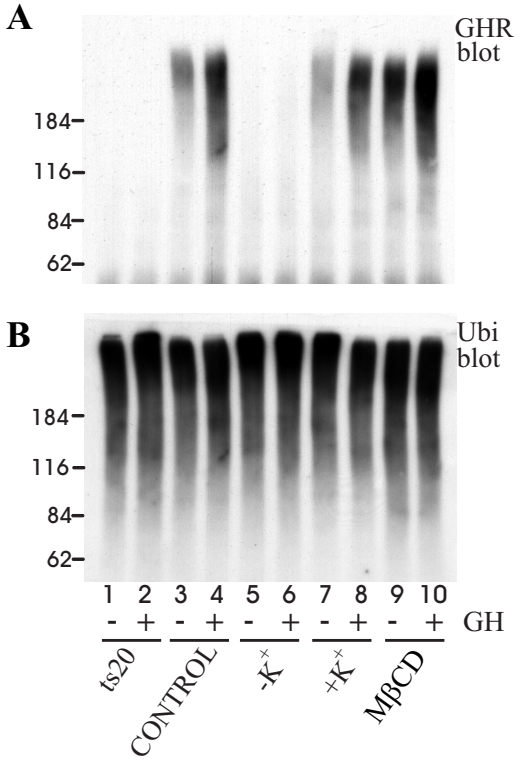
Effect of MβCD on GHR Ubiquitination

To address the question of whether the GHR is ubiquitinated after MβCD treatment, cells were incubated with or without GH, and ubiquitinated proteins were immunoprecipitated and analyzed by Western blotting, as indicated in Fig. 4. Ubiquitinated GHR appeared as high molecular weight species in the top part of the gel (Fig. 4A). Control cells (*lanes* 3 and 4) showed increased GHR ubiquitination on ligand binding. Both in unstimulated and stimulated cells the level of GHR ubiquitination increased when MβCD was present (Fig. 4A, compare *lane* 3 with *lane* 9 and *lane* 4 with *lane* 10). The use of untransfected cells resulted, as expected, in the absence of signal for ubiquitinated GHR (ts20; Fig. 4A, *lanes* 1 and 2). When endocytosis was inhibited by potassium depletion, GHR ubiquitination was almost completely abolished (*lanes* 5 and 6). Ubiquitination was restored to control values by adding 10 mM KCl (*lanes* 7 and 8). Reprobing the blot from Fig. 4A with anti-ubiquitin showed that the amount of immunoprecipitated, ubiquitinated protein in each lane was comparable (Fig. 4B). These results show that the GHR is ubiquitinated at the cell surface before constriction of the coated pit occurs and suggest that assembly of the clathrin coat is a requirement for GHR ubiquitination.

Effect of a Temperature-sensitive Dynamin Mutation on GHR Ubiquitination

To examine the effect of inhibition of clathrin-mediated endocytosis on GHR ubiquitination by an independent method, we used a HeLa cell line expressing a temperature-sensitive mutant of human dynamin, dyn^{TS}. Dyn^{TS} carries a point mutation corresponding to the *Drosophila shibire*^{ts1} allele (30). For this dynamin mutant it has been shown that transferrin internalization is inhibited at the nonpermissive temperature, that

Fig. 4. Effect of potassium depletion and MβCD on GHR ubiquitination.

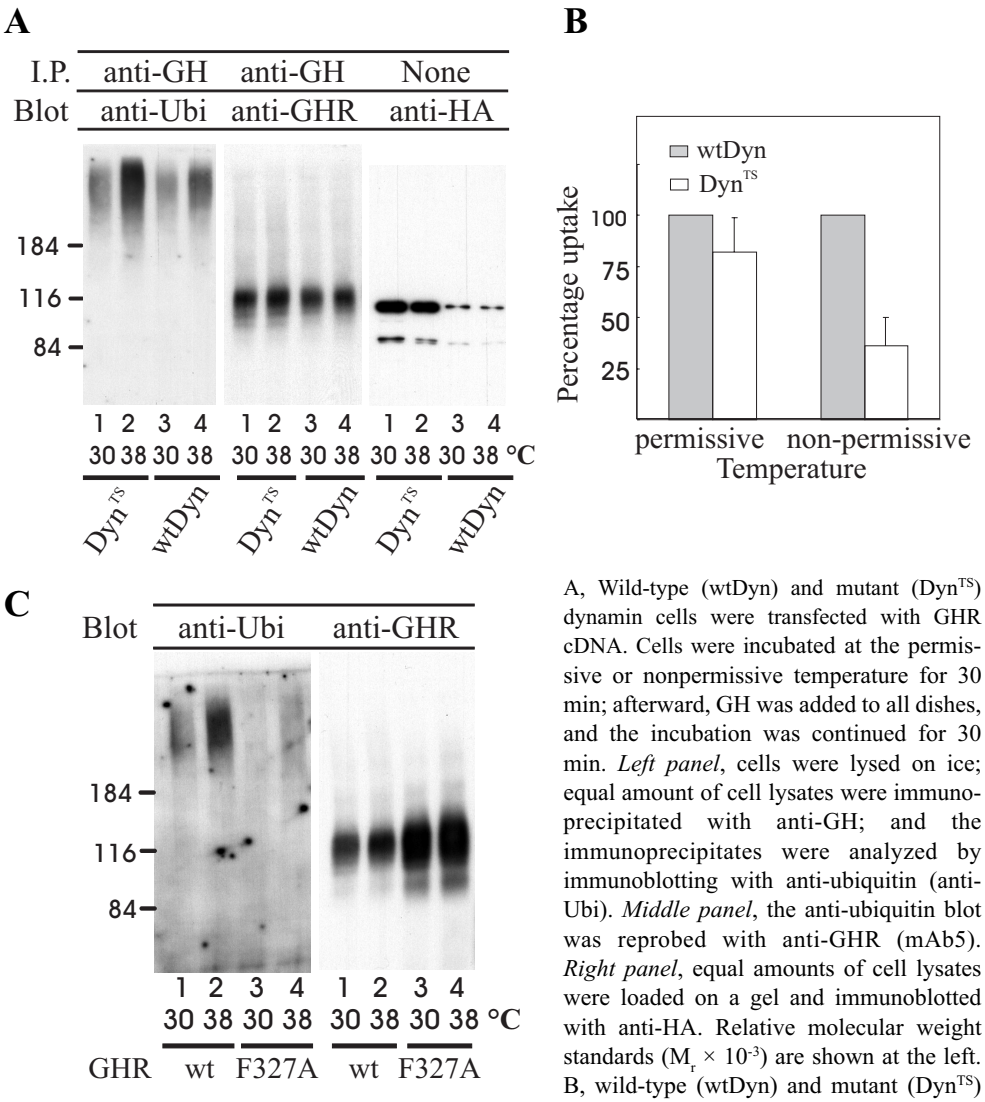


GHR-expressing ts20 cells (*lanes* 3-10) or untransfected cells (ts20; *lanes* 1 and 2) were incubated with medium alone (*lanes* 1-4), in the absence ($-K^+$; *lanes* 5 and 6) or presence ($+K^+$; *lanes* 7 and 8) of potassium after hypotonic shock, or with 10 mM MβCD (*lanes* 9 and 10) for 30 min at 30°C. The incubation was continued for 30 min with (*lanes* 2, 4, 6, 8, and 10, +GH) or without GH. A, cells were lysed with boiling lysis buffer; equal amounts of cell lysates were immunoprecipitated with anti-ubiquitin and analyzed by immunoblotting with anti-GHR (mAb5).

B, the same blot was reprobed with anti-ubiquitin (Ubi). Relative molecular weight standards ($M_r \times 10^{-3}$) are shown at the left.

the phenotype is rapid and reversible, and that invaginated but not constricted coated pits accumulate on the cytoplasmic surface of the plasma membrane. HeLa cells were transiently transfected with wild-type GHR cDNA and incubated in the presence of GH; afterward the GH-GHR complex was immunoprecipitated with anti-GH. Using this approach, only mature GHR species from the cell surface were immunoprecipitated. At the permissive temperature, ubiquitinated protein was detected in both the wild-type and mutant dynamin cells (Fig. 5A, anti-Ubi). After shifting the cells to the nonpermissive temperature, an increase in the amount of ubiquitinated GHR was detected in dyn^{TS} and wild-type dynamin cells (Fig. 5A, anti-Ubi). Ubiquitination of proteins is dynamic with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect the higher endocytotic activity of the cells caused by the elevated temperature or a temperature-dependent shift in balance between ubiquitinating and deubiquitinating enzymes. The increase in amount of ubiquitinated GHR in the dyn^{TS} cells at the nonpermissive temperature was consistently severalfold higher compared with wild-type dynamin cells. The data indicate that GHR ubiquitination precedes GHR endocytosis, which is in agreement with the experiments in which MβCD was used. Reprobing the blot with anti-GHR showed that the amounts of mature receptor expressed in dyn^{TS} and wild-type dynamin cells were comparable (Fig. 5A, anti-

Fig. 5. Effect of overexpression of the dyn^{TS} mutant on GHR ubiquitination and internalization.



A, Wild-type (wtDyn) and mutant (Dyn^{TS}) dynamin cells were transfected with GHR cDNA. Cells were incubated at the permissive or nonpermissive temperature for 30 min; afterward, GH was added to all dishes, and the incubation was continued for 30 min. *Left panel*, cells were lysed on ice; equal amount of cell lysates were immunoprecipitated with anti-GH; and the immunoprecipitates were analyzed by immunoblotting with anti-ubiquitin (anti-Ubi). *Middle panel*, the anti-ubiquitin blot was reprobed with anti-GHR (mAb5). *Right panel*, equal amounts of cell lysates were loaded on a gel and immunoblotted with anti-HA. Relative molecular weight standards ($M_r \times 10^{-3}$) are shown at the left. B, wild-type (wtDyn) and mutant (Dyn^{TS}) dynamin cells were transfected with GHR cDNA. Cells were incubated at the permissive or nonpermissive temperature for 30 min; afterward, ^{125}I-GH was added to all dishes, and the incubation was continued for 30 min. Background signal was measured in the presence of excess unlabeled GH and subtracted. Internalization in the dyn^{TS} mutant was compared with the internalization in the wtDyn; specifically internalized ^{125}I-GH was calculated as a percentage of total cell-associated label and set to 100% for the wtDyn cells. The amount of cell surface expression of GHR was comparable in the two cell lines; mock-transfected cells showed no detectable specific binding of ^{125}I-GH (data not shown). The values are the mean ± S.D. of two experiments performed in duplicate. C, mutant (dyn^{TS}) dynamin cells were transfected with wild-type GHR (wt) or GHR(F327A) cDNA (F327A). Experimental conditions and immunoprecipitations were as described in A. *Left panel*, the Western blot was detected with anti-ubiquitin (anti-Ubi). *Right panel*, the same blot was reprobed with anti-GHR (mAb5).

GHR). The amount of ubiquitinated GHR is only a small fraction of the total amount of GHR bound to GH (see below). The expression of HA-tagged dynamin in the HeLa cells was controlled on a Western blot of cell lysate using anti-HA (Fig. 5A, anti-HA). Both dyn^{TS} and wild-type dynamin cells expressed the HA-tagged dynamin, albeit in different amounts, whereas cells cultured in the presence of tetracycline showed no detectable HA signal (results not shown). To ascertain that indeed GH uptake was inhibited, we measured internalization of ^{125}I-GH. At the permissive temperature the percentage of GH uptake was comparable in the two cell lines, whereas at the nonpermissive temperature GH internalization was strongly inhibited in dyn^{TS} cells compared with wild-type dynamin cells (Fig. 5B). The results demonstrate that overexpression of a dominant negative mutant of dynamin-1 inhibits the clathrin-mediated endocytosis of the GHR. Because we analyzed a complex of proteins immunoprecipitated with anti-GH to monitor ubiquitination of the GHR, the possibility exists that other ubiquitinated proteins coimmunoprecipitate in this complex. To show that the ubiquitination of this complex depends on the GHR, we transfected the internalization-deficient GHR(F327A) mutant in dyn^{TS} cells. Previously, we have shown that this mutant is not ubiquitinated because of a defective ubiquitin-dependent endocytosis motif (15). After immunoprecipitation of the GH-GHR(F327A) complex, almost no ubiquitinated protein was isolated either at the permissive or the nonpermissive temperature (Fig. 5C, *left panel*). This result shows that the ubiquitination of the GH-GHR complex is dependent on an intact ubiquitin-dependent endocytosis motif and that most likely the receptor itself is ubiquitinated, perhaps in complex with other ubiquitinated proteins. Control experiments with mock-transfected cells and incubations without GH showed no signal on the ubiquitin blots (results not shown). As seen in the Fig. 5C, *right panel*, mature GHR and GHR(F327A) were detected in the complex, and virtually no GHR signal is present at the top of the lanes, indicating that only a small percentage of total GHR is ubiquitinated. Most likely, the GHR is ubiquitinated during a very short period, presumably the resident time in the coated pit.

DISCUSSION

In this study two independent methods were used to inhibit clathrin-mediated endocytosis at the level of clathrin-coated vesicle formation. Both methods inhibited the endocytosis of the GHR, resulting in an accumulation of ubiquitinated receptors at the cell surface. A morphological approach detected the GHR in deeply invaginated coated pits in control cells and in flattened clathrin-coated pits in MβCD-treated cells. Disruption of clathrin-mediated endocytosis by hypertonic medium treatment, cytosol acidification, or potassium depletion resulted in the accumulation of nonubiquitinated receptors at the cell surface (Ref. 14 and Fig. 4A). Why is the GHR not ubiquitinated under these conditions? The ubiquitination state of a protein is the result of a dynamic

process of ubiquitination and deubiquitination. Changing the intracellular milieu by depleting potassium or modifying the pH could alter the balance between those two processes. Do the used methods interfere with the ubiquitination machinery itself and cause a complete inhibition of cellular ubiquitination? Analysis of cell lysates from potassium-depleted or hypertonic medium-treated cells showed no reduction in total cellular ubiquitin conjugates but rather an increase in high molecular mass ubiquitinated proteins (Ref. 14 and Fig. 4B). The amount of free ubiquitin under these circumstances as measured with Western blotting was reduced (data not shown). However, cellular cytosol acidification showed increased free and less conjugated ubiquitin (data not shown). Cytosol acidification causes the same precipitation of small clathrin microcages as seen after hypertonicity and potassium depletion (32). Recently, it was shown that after hypertonic treatment or cytoplasmic acidification, free clathrin triskelions within the cytosol are depleted, and all of the clathrin becomes associated with membranes (26). Because the presence of free clathrin triskelions is required for the stabilization of AP-2 coated pit nucleation sites, depletion of clathrin interferes with coated pit formation. Because the methods used have a varying effect on the ratio of free versus conjugated ubiquitin, it is most likely that the inhibition of GHR ubiquitination is the result of the interference with the coated pit formation rather than with ubiquitin conjugation itself.

The observation that GHR ubiquitination coincides with the recruitment of the GHR to clathrin-coated membrane areas suggests that the ubiquitin conjugation system and the endocytosis machinery act together in the endocytosis of the GHR. The earlier observation that ubiquitination of the receptor itself is not important for endocytosis suggests that ancillary proteins might be ubiquitinated or that factors of the ubiquitin conjugation system itself might act as adaptors for the endocytosis machinery. Ubiquitin-protein ligases have been implicated in endocytosis. For the epithelial sodium channel, it was shown that the ubiquitin-protein ligase Nedd4 mediates the down-regulation of the Na^+ channel activity by ubiquitinating the channel, which leads to its endocytosis and degradation (33). The yeast homologue of Nedd4, Npi1/Rsp5, participates via its C2 domain in the endocytosis of Gap1 permease. A truncated Npi1 protein lacking the C2 domain can still promote ubiquitination but not the endocytosis of Gap1 permease, which is consistent with direct participation of Npi1 in endocytosis of the permease (34). Whether an E2/E3 ubiquitin ligase directly serves as an endocytic adaptor for GHR, analogous to the role of β-arrestin for the β2-adrenergic receptor (8) or binding of the ubiquitin conjugation system, results in the interaction with an endocytic adaptor (e.g. AP-2), remains to be established. Recently, GH-dependent association of AP-2 with the chicken GHR was reported (35). Also, a possible role for the ubiquitin polypeptide itself, conjugated to a GHR-associated protein, cannot be excluded, as has been described for Ste2p (36) and Ste3p (37). Ligand-induced ubiquitination was

shown for Eps15, a clathrin-coated pit associated protein that is ubiquitinated on epidermal growth factor (EGF) receptor activation (38). Eps15 is required for clathrin-mediated endocytosis, and perturbation of Eps15 function inhibits receptor-mediated endocytosis of transferrin (39). The biological significance of Eps15 monoubiquitination is not known. Recently, it was shown that polyubiquitination of the EGF receptor occurs at the plasma membrane on ligand-induced activation (40). Inhibition of endocytosis caused by overexpression of mutant dynamin resulted in a transient polyubiquitination of the EGF receptor. The mechanisms for GHR and EGF receptor ubiquitination are probably different. Ubiquitination of the EGF receptor is mediated by Cbl adaptor proteins, and both EGF receptor and Cbl must undergo phosphorylation on specific sites for productive ubiquitination (41), whereas GHR ubiquitination occurs in the absence of GHR tyrosine phosphorylation and is dependent on the ubiquitin-dependent endocytosis motif (20).

The amount of ubiquitinated GHR is very low compared with the total amount of cell surface GHR. The fact that only a small percentage of total GHR is ubiquitinated indicates a coated pit restricted function of the ubiquitin conjugation system. Whether the ubiquitinated GHR is (partially) degraded soon after endocytosis or perhaps rapidly deubiquitinated is not clear at present. A role for deubiquitinating enzymes in regulating endocytosis cannot be excluded, because deubiquitination has been recognized as an important regulatory step (31, 42). Recently, genetic data were presented that support a model whereby the *Drosophila* fat facets deubiquitinating enzyme removes ubiquitin from the product of the *liquid facets* gene. The *liquid facets* locus encodes epsin, a protein involved in clathrin-mediated endocytosis (43). Thus the ubiquitin system may add another layer of complexity to the membrane-sorting machinery at the plasma membrane and regulate, together with the classical endocytosis machinery, the time span of the GHR at the cell surface.

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REFERENCES

1. Mellman, I. (1996) Annu. Rev. Cell Dev. Biol. **12**, 575-625
2. Schmid, S. L. (1997) Annu. Rev. Biochem. **66**, 511-548
3. Chen, W.-J., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. **265**, 3116-3123
4. Collawn, J. F., Stangel, M., Kuhn, L. A., Esekogwu, V., Jing, S. Q., Trowbridge, I. S., and Tainer, J. A. (1990) Cell **63**, 1061-1072
5. Haft, C. R., Klausner, R. D., and Taylor, S. I. (1994) J. Biol. Chem. **269**, 26286-26294
6. Gabilondo, A. M., Hegler, J., Krasel, C., Boivinjahns, V., Hein, L., and Lohse, M. J. (1997) Proc. Natl. Acad. Sci. USA. **94**, 12285-12290
7. Kirchhausen, T. (1999) Annu. Rev. Cell Dev. Biol. **15**, 705-732
8. Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A.W., Keen, J., and Benovic, J. L. (1996) Nature **383**, 447-450
9. Strous, G. J., and Govers, R. (1999) J. Cell Sci. **112**, 1417-1423
10. Hicke, L. (1999) Trends Cell Biol. **9**, 107-112
11. Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) EMBO J. **16**, 6325-6336
12. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. **15**, 3806-3812
13. Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., and Wood, W. I. (1987) Nature **330**, 537-544
14. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) EMBO J. **16**, 4851-4858
15. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. **18**, 28-36
16. van Kerkhof, P., Govers, R., Alves Dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. **275**, 1575-1580
17. Larkin, J. M., Brown, M. S., Goldstein, J. L., and Anderson, R. G. W. (1983) Cell **33**, 273-285
18. Hansen, S. H., Sandvig, K., and VanDeurs, B. (1993) J. Cell Biol. **121**, 61-72
19. Sandvig, K., Olsnes, S., Petersen, O. W., and van Deurs, B. (1987) J. Cell Biol. **105**, 679-689
20. Strous, G. J., van Kerkhof, P., Govers, R., Rotwein, P., and Schwartz, A. L. (1997) J. Biol. Chem. **272**, 40-43
21. Bentham, J., Aplin, R., and Norman, M. R. (1994) J. Histochem. Cytochem. **42**, 103-107
22. Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. **113**, 123-135
23. Liou, W., Geuze, H. J., and Slot, J. W. (1996) Histochem. Cell Biol. **106**, 41-58
24. Ilondo, M. M., Courtoy, P. J., Geiger, D., Carpentier, J., Rousseau, G. G., and de Meyts, P. (1986) Proc. Natl. Acad. Sci. USA. **83**, 6460-6464
25. Ilondo, M. M., Smal, J., DeMeyts, P., and Courtoy, P. J. (1991) Endocrinology **128**, 1597-1602
26. Brown, C. M., and Petersen, N. O. (1999) Biochem. Cell Biol. **77**, 439-448

27. Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H., and Mcgraw, T. E. (1999) Proc. Natl. Acad. Sci. USA. **96**, 6775-6780

28. Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., Vandeurs, B., and Sandvig, K. (1999) Mol. Biol. Cell **10**, 961-974

29. Allevato, G., Billestrup, N., Goujon, L., Galsgaard, E. D., Norstedt, G., and Nielsen, J. H. (1995) J. Biol. Chem. **270**, 17210-17214

30. Damke, H., Baba, T., van der Bliek, A. M., and Schmid, S. L. (1995) J. Cell Biol. **131**, 69-80

31. Hochstrasser, M. (1996) Annu. Rev. Genet. **30**, 405-439

32. Heuser, J. (1989) J. Cell Biol. **108**, 401-411

33. Harvey, K. F., Dinudom, A., Komwatana, P., Jolliffe, C. N., Day, M. L., Parasivam, G., Cook, D. I., and Kumar, S. (1999) J. Biol. Chem. **274**, 12525-12530

34. Springael, J. Y., Decraene, J. O., and Andre, B. (1999) Biochem. Biophys. Res. Commun. **257**, 561-566

35. Vleurick, L., Pezet, A., Kuhn, E. R., Decuypere, E., and Edery, M. (1999) Mol. Endocrinol. **13**, 1823-1831

36. Shih, S. C., Sloper-Mould, K. E., and Hicke, L. (2000) EMBO J. **19**, 187-198

37. Roth, A. F., and Davis, N. G. (2000) J. Biol. Chem. **275**, 8143-8153

38. van Delft, S., Govers, R., Strous, G. J., Verkleij, A. J., and van Bergen en Henegouwen, P. M. (1997) J. Biol. Chem. **272**, 14013-14016

39. Benmerah, A., Bayrou, M., Cerfbensussan, N., and Dautry-Varsat, A. (1999) J. Cell Sci. **112**, 1303-1311

40. Stang, E., Johannessen, L. E., Knardal, S. L., and Madshus, I. H. (2000) J. Biol. Chem. **275**, 13940-13947

41. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell **4**, 1029-1040

42. Chung, C. H., and Baek, S. H. (1999) Biochem. Biophys. Res. Commun. **266**, 633-640

43. Cadavid, A. L., Ginzel, A., and Fischer, J. A. (2000) Development **127**, 1727-1736

Chapter III

ENDOCYTOSIS AND DEGRADATION OF THE GROWTH HORMONE RECEPTOR ARE PROTEASOME-DEPENDENT

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ABSTRACT

The ubiquitin conjugation system is involved in ligand-induced endocytosis of the growth hormone receptor (GHR) via a cytosolic 10-amino acid ubiquitin-dependent endocytosis motif. Herein, we demonstrate that the proteasome is also involved in growth hormone receptor down-regulation. Ligand-induced degradation was blocked in the presence of specific proteasomal inhibitors. In addition, growth hormone (GH) internalization was inhibited, whereas the transferrin receptor cycle remained unaffected. A truncated GHR entered the cells independent of proteasome action. In addition, we show that GH internalization is independent of the presence of lysine residues in the cytosolic domain of the receptor, whereas its internalization can still be inhibited by proteasomal inhibitors. Thus, GHR internalization requires proteasome action in addition to an active ubiquitin conjugation system, but ubiquitination of the GHR itself seems not to be required.

Abbreviations used:

GHR, growth hormone receptor; GH, growth hormone; CHO-ts20, Chinese hamster cell line carrying a temperature-sensitive ubiquitin activation enzyme E1; MEMα, minimum Eagle's medium; PBS, phosphate-buffered saline.

INTRODUCTION

The growth hormone receptor (GHR) is a mammalian plasma membrane protein whose internalization is mediated by the ubiquitin conjugation system (1). In particular, a 10-amino acid motif including Phe-327 within the GHR cytosolic tail (the UbE motif) is involved in both GHR ubiquitination and ligand-induced receptor endocytosis. In addition, ubiquitination of the GHR itself is not necessary for ligand internalization (2). The receptor has a short half-life (3-5), and the degradation occurs within the lysosome (3, 6). However, it has been suggested that the GHR is also transported to the nucleus (7), to detergent-insoluble membrane domains (8), and back to the plasma membrane (9). GHR signaling is initiated at the plasma membrane when two receptors are dimerized by a single GH molecule (10). This dimerization induces recruitment and binding of the tyrosine kinase JAK2, resulting in the activation of various signal transduction pathways (reviewed in Ref. 11). The GHR was initially found to be ubiquitinated upon amino acid sequencing of the receptor from rabbit liver (12). Binding of GH stimulates ubiquitination, internalization, and degradation of the receptor. In a Chinese hamster cell line carrying a temperature-sensitive ubiquitin activation enzyme E1 (CHO-ts20; see Ref. 13), inactivation of E1 results in an accumulation of non-ubiquitinated GHRs at the plasma membrane, whereas internalization of the transferrin receptor is unaffected (1, 14). These data show that GHR ubiquitination and internalization are related.

Degradation of cytosolic proteins is mainly carried out by the 26S proteasome. The ubiquitin conjugation system selects and targets the proteins for proteasomal degradation (15). In a growing number of cases the ubiquitin conjugation system seems to be involved in the selection steps directly preceding endocytosis at the plasma membrane. In yeast the α-factor receptor Ste2p (16), the Ste6 peptide transporter (17), Gap1p amino acid permease (18), Gal2p galactose transporter (19), Fur4 uracil permease (20), and Pdr5 (21), a multidrug transporter, all undergo ubiquitin-dependent endocytosis. Inferred from genetic studies, proteasome activity is neither necessary for ubiquitin-dependent endocytosis nor for vacuolar degradation in yeast. In mammalian cells, studies with proteasomal inhibitors suggest a role for the proteasome in the degradation of the Met tyrosine kinase receptor (22), the platelet-derived growth factor receptor (23), the low density lipoprotein receptor (24), and the mannose phosphate receptor (25).

Down-regulation of signal transducing membrane receptors is a part of the highly programmed cascade of events leading both to extinction of the signaling pathway(s) and to rapid degradation of the primary messengers, the receptor and its ligand (5, 26-29). In the absence of ligand the half-life of GHR is approximately 1-2 h depending on the cell system used. The assumption is that this is mainly due to a proteolytic cleavage in the extracellular domain of the GHR resulting in soluble GH-binding proteins (30); the fate of the cytosolic tail in this process is unknown. If ligand is present, a completely different scenario follows; two GHR polypeptides dimerize, they are phosphorylated by

the tyrosine kinase JAK2 and ubiquitinated, and the complex is then endocytosed. As the ubiquitin conjugation system acts generally in concert with the 26S proteasome, we examined the effect of proteasomal inhibitors on GH uptake via wild-type GHR and a receptor truncated at amino acid residue 369 in CHO-ts20 cells (1). The data show that specific proteasomal inhibitors block GH uptake via the full-length GHR, whereas a truncated receptor can endocytose undisturbed. Nonetheless, the ubiquitin conjugation system remains necessary for the truncated receptor to be endocytosed (14). Evidence is provided that proteasomal action does not require ubiquitination of the receptor itself.

MATERIALS AND METHODS

Cells and Antibodies

A polyclonal antibody to the cytosolic tail was raised in rabbits against the membrane-proximal amino acid residues 271-318 (anti-T) (see Fig. 1) as described in Ref. 1; antibody (Mab5) recognizing the luminal part of the GHR was from AGEN Inc., Parsippany, NJ. Antiserum specific for protein-ubiquitin conjugates was a generous gift from Dr. A. Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). CHO-ts20 was transfected with both the full-length rabbit GHR cDNA sequence and a cDNA encoding GHR truncation 1-369 (1, 13). 10 mM sodium butyrate was added to the cells 18 h before use to increase GHR expression (14).

Mutagenesis and Transfection

cDNA encoding GHR truncation mutant GHR1-369 was constructed by introducing a stop codon at the proper position within the GHR cDNA. For this GHR truncation, a polymerase chain reaction was performed using a 5'-oligonucleotide containing a *NcoI* restriction site corresponding to the *NcoI* site in the cDNA of the transmembrane region of the GHR together with a 3'-oligonucleotide containing a *KpnI* restriction site, a stop codon, and overlapping sequences at position 369 within the cDNA, encoding the intracellular domain of the GHR. The polymerase chain reaction product was cut by *NcoI* and *KpnI* and ligated into a pGEM3Z-GHR construct. The truncated GHR cDNA (see Fig. 1) was subcloned into the pcDNA3 vector (Invitrogen). cDNA of mutants GHR F327A and GHR1-399 K271-362R were constructed as described (2, 14).

GH Binding and Internalization

^{125}I-human GH was prepared using chloramine T (1). For internalization studies, cells were grown in 35-mm dishes, washed with MEMα supplemented with 20 mM Hepes and 0.1% bovine serum albumin, and incubated for 1 h at 30°C in MEMα/Hepes. ^{125}I-GH (8 nM) was bound on ice for 120 min in the absence or presence of excess unlabeled GH, and the cells were washed free of unbound GH and incubated for 0-30 min at 30°C. If indicated, lactacystin (20 μM) or its β-lactone (20 μM), MG132 (20 μM), and carboxy benzyl leucyl-leucyl-leucinevinylsulfone (20 μM), dissolved in either ethanol or dimethyl sulfoxide, or vehicle only were added 1 h before the start of the experiment. Membrane-associated GH was removed by acid wash (0.15 M NaCl, 0.1% bovine serum albumin, 0.05 M glycine, pH 2.5), and internalized GH was determined by measuring the radioactivity after solubilization of the acid-treated cells by 1 M NaOH.

Metabolic Labeling

For metabolic labeling, the cells were incubated in methionine-free MEM for 20 min and then [^{35}S]methionine (Tran-^{35}S Label, 1.85 MBq/ml, 40 TBq/mmol, ICN Biomedicals, Costa Mesa, CA) was added and the incubation was continued for 20 min; the radioactivity was chased in the presence

of MEMα containing 0.05 mM unlabeled methionine, 0.18 μg/ml GH, 0.1% bovine serum albumin, and the appropriate proteasomal inhibitor. Cells were lysed in boiling buffer (see below). The radioactivity was determined using a Molecular Dynamics PhosphorImager.

Cell Lysis and Western Blotting

At the end of the incubation, cells were washed and immediately lysed in boiling buffer containing 1% SDS in PBS. We used this protocol for all of our experiments to ascertain that no GHR or the derived degradation products were lost because of poor solubility of GH-GHR complexes, as reported by Goldsmith et al. (8). Equal aliquots of the cell extracts were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as described (14). For detection we used the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

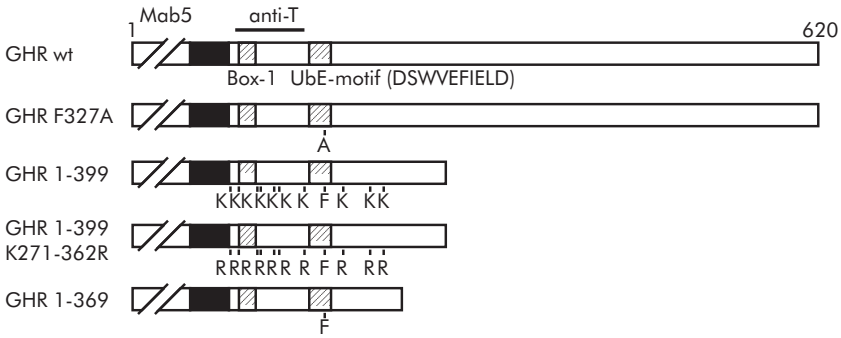
Immunoprecipitations

Immunoprecipitations were performed as described previously (1). Extracts from cells lysed in boiling buffer were subjected to immunoprecipitation in PBS containing 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 μM MG132. The lysates were incubated with anti-ubiquitin or anti-GHR antiserum as indicated in the experiments represented in the figures for 2 h on ice. Immune complexes were isolated by the use of protein A-agarose beads (Repligen Co., Cambridge, MA).

Microscopy

Cy3-GH and Cy3-transferrin were prepared using a FluoroLink Cy3 label kit according to the supplier's instructions (Amersham Pharmacia Biotech). Cells grown on coverslips were incubated for 60 min in MEMα supplemented with 20 mM Hepes at 30°C and for 30 min with Cy3-GH (1 μg/ml) or Cy3-transferrin (20 μg/ml). Cells were washed with PBS to remove unbound label and fixed for two h in 3% paraformaldehyde in PBS. Confocal laser scanning microscopy was performed using a Leica TCS 4D system.

Fig. 1. Schematic representation of the wild-type and mutant GHRs



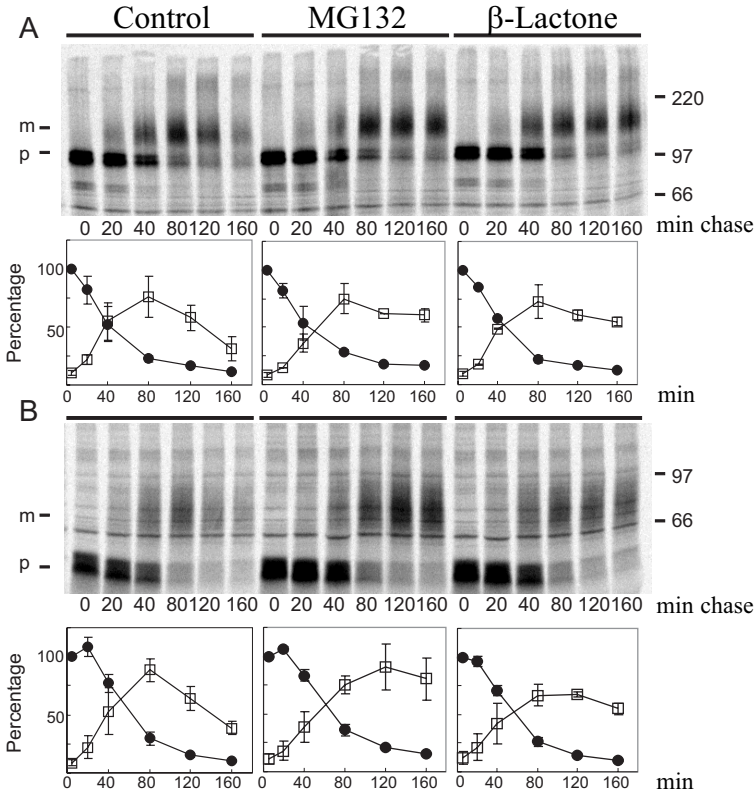
For GHR mutants truncated at residue 399, all cytoplasmic lysines, lysine mutations, and Phe-327 are indicated. For GHR mutants truncated at residue 369, only Phe-327 is indicated. A black square represents the transmembrane domain. Hatched squares represent box-1 (site for JAK2 binding) and the UbE motif (site for interaction with the ubiquitin system). The antibody specificities are indicated at the top of the figure. Mab5 is a monoclonal antibody raised against GH-binding proteins; anti-T was raised against a GST-fusion protein to the GHR peptide 271-318.

RESULTS

Effect of Proteasomal Inhibitors on GHR Turnover

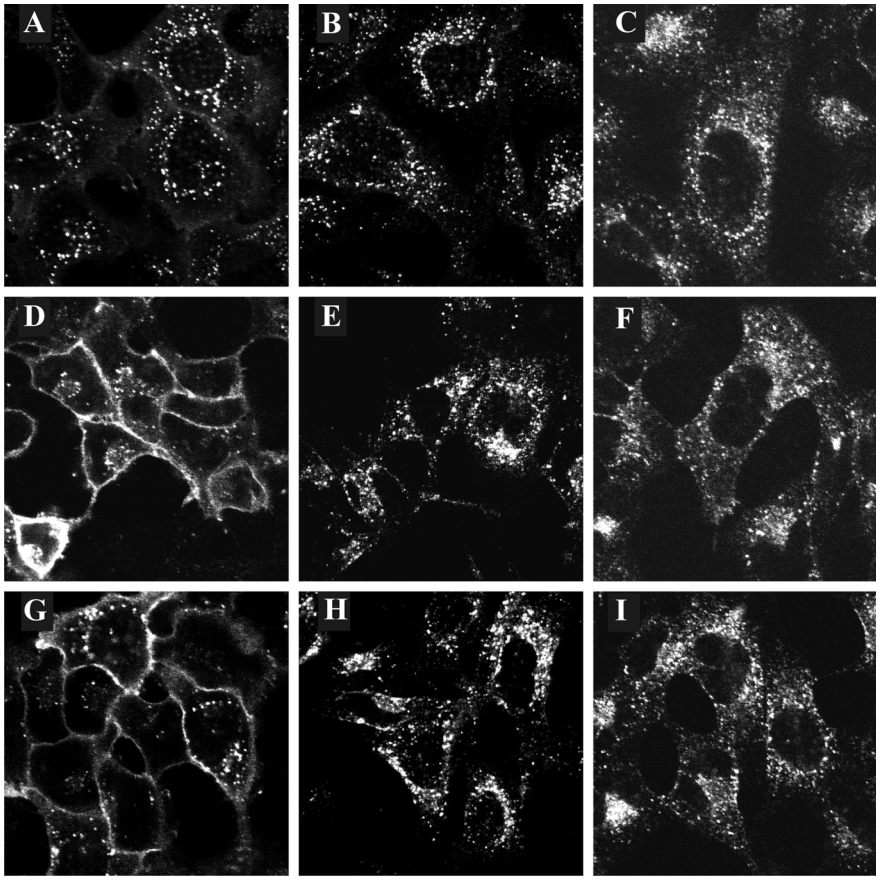
Initially, we investigated the life cycle of the GHR at the permissive temperature (30°C) in the presence of GH in CHO-ts20 cells transfected with GHR cDNA using pulse-chase labeling with [^{35}S]methionine (see Fig. 2A). The receptor is synthesized as a 110-kDa glycoprotein precursor (double band) and, upon "complex glycosylation" in the Golgi complex, converted to a 130-kDa mature species. The nature of the double

Fig. 2. Effect of proteasomal inhibitors on [^{35}S]methionine-labeled GHRs



CHO-ts20 cells were labeled with [^{35}S]methionine for 20 min and chased in MEMα supplemented with 0.1% bovine serum albumin and 0.18 μg/ml GH for the time periods indicated. GHR was immunoprecipitated using anti-T. MG132 or β-lactone were present 60 min before the start and throughout the pulse-chase period. A, CHO-ts20 cells expressing the full-length GHR. m, mature (130 kDa of GHR); p, precursor (110 kDa). B, CHO-ts20 cells expressing the GHR1-369. m, mature (85 kDa of GHR); p, precursor (60 kDa). Relative molecular weight standards (M_r x 10^{-3}) are shown to the right. The amounts of radioactivity were determined using ImageQuant (Molecular Dynamics) and were expressed as percentages of the radioactivity incorporated in the precusor GHR after the pulse labeling.●, precursor GHR; □, mature GHR. The values represent the mean ± S.D. of two different experiments.

Fig. 3. Effect of proteasomal inhibitors on Cy3-GH and Cy3-transferrin endocytosis



CHO-ts20 cells, expressing either wild-type (A, D, G, C, F, and I), or truncated GHR1-369 (B, E, and H) were incubated with vehicle (A-C), 20 μM MG132 (D-F), or 20 μM lactacystin (G-I) for 1 h at 30°C; then Cy3-GH (A, B, D, E, G, and H) or Cy3-transferrin (C, F, and I) were added for 30 min, and the cells were washed, fixed, and the fluorescence was visualized by confocal microscopy. No uptake was observed when excess unlabeled ligand was added. Virtually no label was visible when the cells in D and G were treated at pH 2.5 before fixation (not shown).

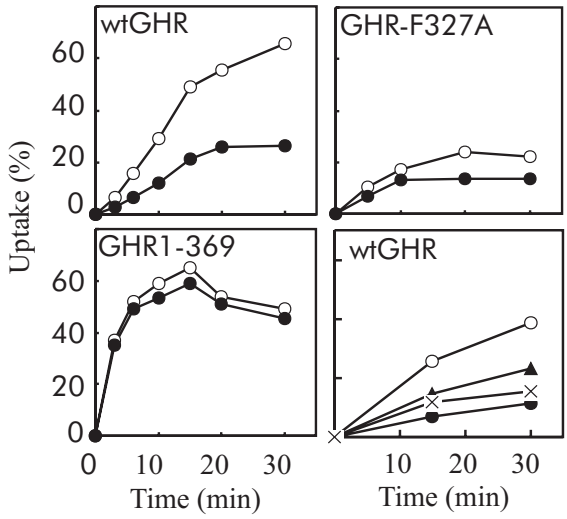
band is unknown. Mature GHR was detectable by 20 min of chase and was maximal at 80 min, after which rapid degradation occurred. When the pulse-chase was performed in the presence of the specific proteasomal inhibitors MG132 or the more membrane permeable β-lactone analogue of lactacystin (31, 32), the amount of labeled mature GHR was hardly decreased after 160 min of chase. Both inhibitors did not affect the endoplasmic reticulum to Golgi transport, as is concluded from the kinetics by which the GHR precursor is converted to the mature GHR. When the experiment was performed with a cell line expressing the GHR with the cytosolic tail truncated after amino acid residue 369 (GHR1-369, Fig. 1), basically the same results were obtained (Fig.

2B). In the presence of *clasto*-lactacystin β-lactone the amount of GHR1-369 that became mature after 80 min was diminished, but the kinetics of degradation between 80 and 160 min of chase were similar to the results with MG132. These results demonstrate that the mature GHR has a relatively short half-life and that the proteasome plays an important role in GH-induced degradation.

Effect of Proteasomal Inhibitors on GHR Endocytosis

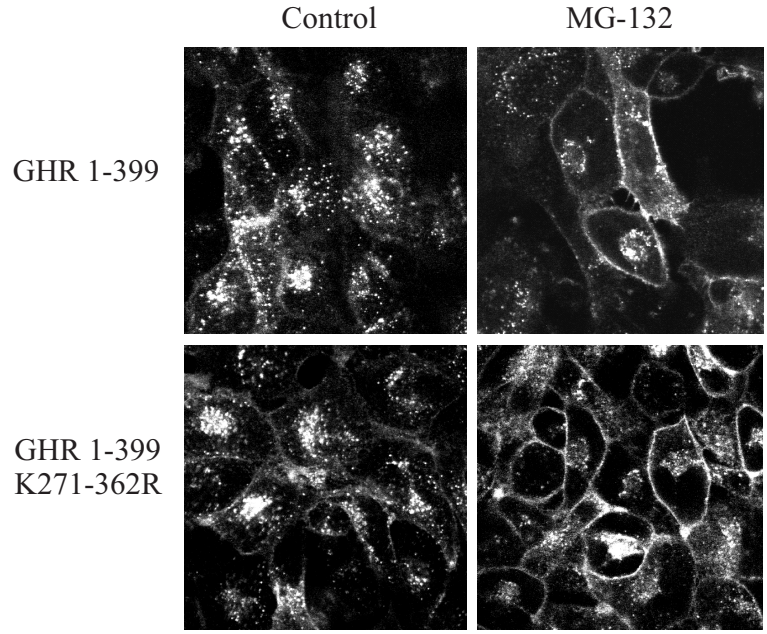
Ligand-induced endocytosis of the growth hormone receptor occurs only if the ubiquitin conjugation system is functional. An issue is thus whether at the same time proteasomal action is required. To test this we used the GHR-transfected CHO-ts20 cells expressing approximately 10^6 ligand-binding sites per cell (14). Incubation for 30 min in the presence of Cy3-labeled GH resulted in abundant fluorescent label in endosomal and lysosomal compartments (Fig. 3A). If the cells were treated with MG132 (Fig. 3D) or lactacystin (Fig. 3G) little label was present intracellularly. The same results were obtained when the cells were treated with carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone or *clasto*-lactacystin β-lactone (not shown). To ascertain that these proteasomal inhibitors did not cause pleiotropic effects on the receptor-mediated endocytic machinery we used Cy3-labeled transferrin under identical conditions (Fig. 3, C, F, and I); no inhibition of transferrin uptake was observed. We next addressed the question as to whether the proteasome acts directly or indirectly on the GHR. Experiments with Chinese hamster cells expressing a GHR1-369 show that the same proteasomal inhibitors as used for the full-length GHR do not affect GH endocytosis (Fig. 3, B, E, and H). Thus, removal of a portion of the cytosolic tail is sufficient to uncouple proteasomal action and GH-dependent endocytosis.

Fig. 4. Effect of proteasomal inhibitors on uptake kinetics of ^{125}I-GH



CHO-ts20 cells were incubated with or without inhibitors for 1 h at 30°C and put on ice for 2 h with ^{125}I-GH. The cells were then incubated at 30°C as indicated. Background label was determined in the presence of excess unlabeled GH and subtracted. The amounts of internalized ^{125}I-GH are plotted as a percentage of the cell-associated radioactivity at the start of incubation. For the experiments in the *lower panel* ^{125}I-GH uptake was measured without prior binding on ice; this explains the different uptake kinetics as compared with the *upper panels*.
○, control (1% ethanol); ●, MG132; ▲, lactacystin; X, *clasto*-lactacystin β-lactone.

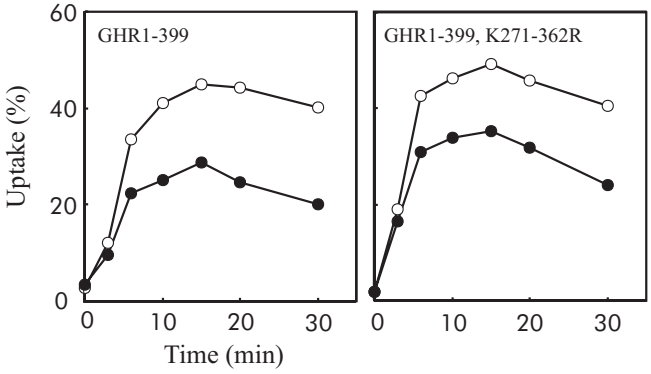
Fig. 5. Internalization of ligand by a GHR that contains no potential ubiquitin conjugation sites



CHO-ts20 cells expressing GHR truncation mutant 1-399 or truncation 1-399 K271-362R were incubated with Cy3-GH for 1 h at 30°C without (Control) or with MG-132. Cy3-GH was visualized by confocal microscopy.

To confirm and quantify the effect of the proteasomal inhibitors we measured the uptake of ^{125}I-GH in kinetic experiments (Fig. 4). Cells were pre-treated with the inhibitors, ^{125}I-GH was bound on ice, and cells were incubated at 30°C for various periods of time. After cooling to 0°C and washing the cells at a low pH value to remove label from the cell surface, uptake was determined. Again, GH uptake was inhibited by MG132. To avoid secondary effects such as receptor recycling and/or early ligand degradation, we measured the initial uptake kinetics. To ascertain the involvement of proteasomes in this process we used several other proteasomal inhibitors; lactacystin reduced the uptake somewhat less effectively, probably because of its poor cell-permeant properties, whereas *clasto*-lactacystin β-lactone was almost as effective as MG132 (Fig. 4, *lower panel*). As expected, uptake via the GHR1-369 was not affected by the proteasomal inhibitors. It was also observed that the truncated receptor is internalized much more rapidly than the full-length GHR. To determine background uptake values we used the full-length GHR in which the Phe-327 was mutated to alanine. This mutation abolishes the ubiquitin-dependent uptake of GH. As seen in Fig. 4 (GHR-F327A), this receptor shows a low GH uptake that was not further decreased in the presence of MG132. The level of GH uptake via the GHR F327A mutant is similar to that via the wild-type GHR in the presence of MG132, indicating that proteasomal inhibitors affect

Fig. 6. Quantitation of GH-uptake by lysine-less GHR



For details, see the legend to Fig. 4.

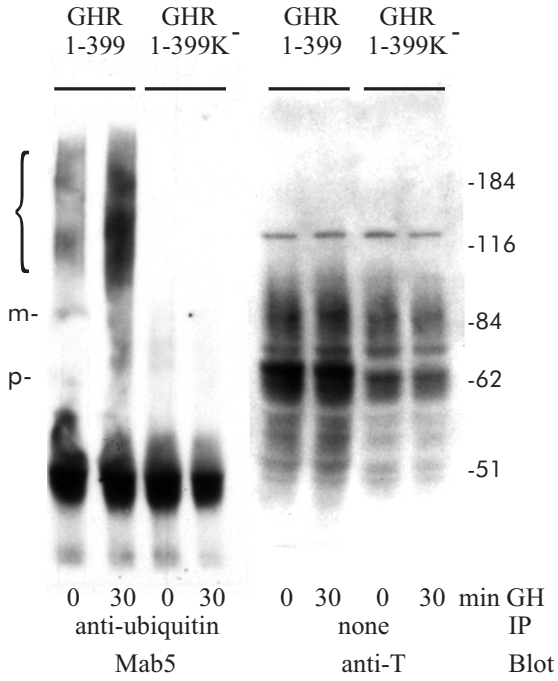
the ubiquitin system-dependent GH uptake. These results show that ubiquitin system-dependent uptake via the full-length receptor is fully blocked in the presence of proteasomal inhibitors.

Ubiquitination of the GHR Itself Is Not Required

To investigate the role of ubiquitination in the proteasome-dependent endocytosis of the GHR, we addressed whether ubiquitination of the receptor was required. We constructed a GHR in which all cytoplasmic lysine residues were mutated to arginines. Because internalization of ligand by GHR1-399 is as dependent on an intact ubiquitin conjugation system as the wild-type GHR, we used a construct for these experiments in which all 10 cytoplasmic lysine residues were mutated (GHR1-399, K271-362R) (18). Both truncations endocytose GH in an ubiquitin system-dependent fashion (2). Fig. 5 shows that MG132 does inhibit ligand uptake for the GHR1-399. In addition it shows that, even if the GHR tail does not contain attachment sites for ubiquitin, endocytosis is inhibited by the proteasomal inhibitor. To quantify the fluorescence experiments ^{125}I-GH uptake was measured in these cells in the presence and absence of the proteasomal inhibitor MG132. Although the inhibitory effect of MG132 is less than for the full-length GHR, it is clear that MG132 inhibits ligand uptake (Fig. 6). An important difference between GH-uptake by the full-length and the truncated GHR is that the initial rate of uptake is higher as the cytosolic tail is shorter. This is certainly the case for truncations up to the di-leucine motif at position 349 (33). An explanation is still nonexistent. To show that ubiquitin attachment is indeed absent, we immunoprecipitated the two truncated GHRs with an antiserum against ubiquitin after stimulation with GH for 30 min and detected the blots with anti GHR. As seen in Fig. 7, GH induces a strong increase in the amount of ubiquitinated GHR1-399, whereas no signal is detectable for the lysine-less GHR1-399. The amounts of mature receptors in both cell lines is approximately the same, as shown in Fig. 7 (*right panel*), in which cell lysates were immunoblotted with anti-GHR (anti-T). These results allow two conclusions: 1) the

inhibitory effect of proteasomal inhibitors resides in a 30-amino acid segment between amino acid residues 369 and 399, and 2) ubiquitination of the (truncated) GHR is not required for the proteasome-dependent GHR internalization.

Fig. 7. Ubiquitination assay of the lysine-less GHR



Cells expressing GHR1-399 or the lysine-less counterpart (GHR1-399, K271-362R), designated as GHR1-399K$^-$) were incubated with or without GH for 30 min at 30°C. *Left panel*, immunoprecipitations (IP) were performed with anti-ubiquitin and analyzed by immunoblotting (Blot) using anti-GHR (Mab5). *Bracket* indicates ubiquitinated GHR; p, precursor GHR1-399; m, mature GHR1-399. *Right panel*, cell lysates (equal amounts of protein) were immunoblotted with anti-T.

DISCUSSION

It is generally accepted that the ubiquitin/proteasome system is involved in selective degradation of cytosolic and nuclear proteins (15). At the cytosolic face of the endoplasmic reticulum the ubiquitin/proteasome system is involved in degradation of misfolded endoplasmic reticulum proteins (34, 35). Previously, we have shown that GHR endocytosis requires an intact ubiquitin system and that GH internalization is accompanied by GHR ubiquitination (1, 14). Endocytosis of the GHR occurs via clathrin-coated pits (28). This process requires interaction between adapter proteins such as AP2 or β-arrestin and clathrin as well as endocytosis motifs within the cytosolic tail of membrane proteins. The tyrosine-based motif YXXϕ (where X is any amino acid and ϕ is an amino acid with a bulky hydrophobic group) is involved in the endocytosis of many transmembrane proteins such as the receptors for low density lipoprotein, transferrin, and asialoglycoproteins (36-38). Internalization of the insulin and β2-adrenergic receptors is mediated by the di-leucine endocytosis motif (39, 40). GHR contains an endo-

cytosis-competent di-leucine motif (DTDRLL), but this acts only if the receptor is truncated immediately after the motif (33). Moreover, ligand-induced GHR endocytosis fully depends on the UbE motif (2). Herein we present evidence that proteasomal action is required for receptor down-regulation in addition to the ubiquitin system and the clathrin-coated pit machinery.

What is the substrate for the proteasome? The most obvious target is the GHR tail itself. This is indicated by the fact that a GHR truncated at position 369 can enter the cells if proteasomal action is blocked. In this scenario the GHR can only be recognized by the endocytic machinery if the tail is cut. In favor of this idea is the fact that attempts to detect full-length GHR intracellularly failed (not shown). Another possibility is that the GHR binds, via the amino acid sequence 369-399, to a factor that has to be removed by the proteasome before endocytosis can proceed. This would explain why ubiquitination of the GHR *per se* is not required.

The amino acid sequence starting at amino acid 365 (DSGRTS) is homologous to an amino acid sequence of human immunodeficiency virus, type I-encoded gene, the membrane protein Vpu. Vpu is a target for the ubiquitin system in the rough endoplasmic reticulum and acts as an intermediary in the degradation of CD4 (41). In the truncated receptor GHR1-369 this motif is disrupted, which would provide a third possible scenario: the F-box protein βTrCP or an analogous protein would bind via its WD40 domain to the motif in the full-length receptor, after which proteasomal action could start. Whatever scenario is correct, it is unlikely that the proteasome truncates the receptor beyond residue 334, because this would disable the UbE motif and prevent endocytosis (14).

Ubiquitination of the GHR is not required for proteasomal action. This phenomenon was reported before for GHR endocytosis via the UbE motif. If truncated receptors like GHR1-369 and GHR1-334 are used endocytosis still depends on an active ubiquitin conjugation system, but their ubiquitination is not required (2). Moreover, proteasomal inhibitors do not affect GH uptake via these short GHRs (Fig. 4 and data not shown). Thus, both proteasomal action and UbE-induced events precede endocytosis (i.e. selection into the coated pits and coated vesicle formation). Collectively, the data indicate that the action of proteasomes precedes the events directed via the UbE motif, although it cannot be excluded that the latter might control the action of the proteasome. For both events, ubiquitination of the GHR tail is not required. A possible explanation is that the ubiquitin ligases involved contain ubiquitin-like proteins as reported for the E3 that is involved in von Hippel-Lindau tumor suppressor function (42).

Once established that proteasomal inhibitors prevent endocytosis, it is not surprising that the half-life of the GHR is prolonged under such conditions. Previously we and others have shown that the GHR and its ligand are degraded within the lysosome (1, 3, 6). This is certainly the case for the luminal domain of the receptor. Our present data

strongly suggest that at least a portion of the GHR is a target for the proteasome system. It has been proposed that the lysosomal and the ubiquitin/proteasome pathway may cooperate in degrading some tyrosine kinase receptors (23). Proteasomal action has been reported to be involved in the turnover of other receptors like the Met tyrosine kinase receptor (22), the platelet-derived growth factor-β receptor (43), the low density lipoprotein receptor (24), and the mannose phosphate receptor (25). Endocytosis of the latter three receptors is not dependent on the ubiquitin/proteasome system. More similarities exist between the degradation of the Met tyrosine kinase receptor and the GHR. Like the GHR, the Met tyrosine kinase receptor undergoes a ligand-independent proteolytic cleavage in its extracellular domain (44), and the receptor is ubiquitinated upon ligand binding (22). Our observation that specific proteasomal inhibitors inhibit degradation of wild-type GHR (at the cell surface) and truncated GHR1-369 (at the cell surface and in endosomes) suggests that the proteasome inhibitors affect both endocytosis and membrane sorting to the lysosomes. One striking difference between the two receptors is that ubiquitination of the Met receptor is increased in the presence of proteasomal inhibitors, whereas ubiquitination of the GHR is decreased under all conditions that block its endocytosis (14), including proteasomal inhibitors (not shown). As the Met tyrosine kinase receptor lacks the UbE motif that is instrumental in ubiquitin-dependent GHR endocytosis, it is tempting to speculate that stabilization of the Met tyrosine kinase receptor by proteasomal inhibitors is due to an intracellular block e.g. in a membrane-sorting step en route to the lysosomes, analogous to the mechanism that connects cbl to the degradation of epidermal growth factor and platelet-derived growth factor receptors (45, 46).

Although the present findings apply to the GHR, there are indications that the ubiquitin/proteasome system is involved in regulation of the residence time at the cell surface of other membrane proteins as well. The residence time of the sodium channel protein ENaC is regulated by the ubiquitin system (47). Many signaling membrane receptors e.g. the Met tyrosine kinase receptor (22), the TCRζ-chain (48), the c-Kit receptor (49), epidermal growth factor receptor (50), and prolactin receptor (51) are all ubiquitinated upon activation. Thus, the ubiquitin/proteasome system may set the timer for cell surface residency and life time for a selected number of key regulatory cell surface molecules.

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REFERENCES

1. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. **15**, 3806-3812
2. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. **18**, 28-36
3. Murphy, L. J., and Lazarus, L. (1984) Endocrinology **115**, 1625-1632
4. Baxter, R. C. (1985) Endocrinology **117**, 650-655
5. Roupas, P., and Herington, A. (1988) Mol. Cell. Endocrinol. **57**, 93-99
6. Yamada, K., Lipson, K. E., and Donner, D. B. (1987) Biochemistry **26**, 4438-4443
7. Lobie, P. E., Wood, T. J. J., Chen, C. M., Waters, M. J., and Norstedt, G. (1994) J. Biol. Chem. **269**, 31735-31746
8. Goldsmith, J. F., Lee, S. J., Jiang, J., and Frank, S. J. (1997) Am. J. Physiol. **36**, E932-E941
9. Ilondo, M. M., Vanderschueren Lodeweyckx, M., Courtoy, P. J., and de Meyts, P. (1992) Endocrinology **130**, 2037-2044
10. Cunningham, B. C., Ultsch, M., deVos, A. M., Mulkerrin, M. G., Clauser, K. R., and Wells, J. A. (1991) Science **254**, 821-825
11. Carter Su, C., and Smit, L. S. (1998) Recent Prog. Horm. Res. **53**, 61-83
12. Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, L., Waters, M. J., and Wood, W. I. (1987) Nature **330**, 537-544
13. Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A., and Marcus, M. (1988) J. Biol. Chem. **263**, 15726-15731
14. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) EMBO J. **16**, 4851-4858
15. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. **67**, 425-479
16. Hicke, L., and Riezman, H. (1996) Cell **84**, 277-287
17. Kölling, R., and Hollenberg, C. P. (1994) EMBO J. **13**, 3261-3271
18. Hein, C., and Andre, B. (1997) Mol. Microbiol. **24**, 607-616
19. Horak, J., and Wolf, D. H. (1997) J. Bacteriol. **179**, 1541-1549
20. Galan, J. M., Moreau, V., Andre, B., Volland, C., and Haguenauer-Tsapis, R. (1996) J. Biol. Chem. **271**, 10946-10952
21. Egner, R., and Kuchler, K. (1996) FEBS Lett. **378**, 177-181
22. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vandewoude, G. F. (1997) Mol. Cell. Biol. **17**, 799-808
23. Mori, S., Tanaka, K., Omura, S., and Saito, Y. (1995) J. Biol. Chem. **270**, 29447-29452
24. Miura, H., Tomoda, H., Miura, K., Takishima, K., and Omura, S. (1996) Biochem. Biophys. Res. Commun. **227**, 684-687
25. Breuer, P., and Braulke, T. (1998) J. Biol. Chem. **273**, 33254-33258
26. Argetsinger, L. S., and Carter Su, C. (1996) Physiol. Rev. **76**, 1089-1107
27. Gorin, E., and Goodman, H. M. (1985) Endocrinology **116**, 1796-1805
28. Lesniak, M. A., and Roth, J. (1976) J. Biol. Chem. **251**, 3720-3729

29. Hackett, R. H., Wang, Y.-D., Sweitzer, S., Feldman, G., Wood, W. I., and Larner, A. C. (1997) J. Biol. Chem. **272**, 11128-11132

30. Baumann, G., Stolar, M. W., Amburn, K., Barasano, C. P., and deVries, B. C. (1986) J. Clin. Endocrinol. Metab. **62**, 134-141

31. Bogyo, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L., and Ploegh, H. (1997) Proc. Natl. Acad. Sci. USA. **94**, 6629-6634

32. Craiu, A., Gaczynska, M., Akopian, T., Gramm, C. F., Fenteany, G., Goldberg, A. L., and Rock, K. L. (1997) J. Biol. Chem. **272**, 13437-13445

33. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1998) J. Biol. Chem. **273**, 16426-16433

34. Wiertz, E. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L. (1996) Cell **84**, 769-779

35. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) Science **273**, 1725-1728

36. Chen, W.-J., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. **265**, 3116-3123

37. Collawn, J. F., Stangel, M., Kuhn, L. A., Esekogwu, V., Jing, S. Q., Trowbridge, I. S., and Tainer, J. A. (1990) Cell **63**, 1061-1072

38. Spiess, M. (1990) Biochemistry **29**, 10009-10018

39. Haft, C. R., Sierra, N. D. L., Hamer, I., *et al.* (1998) Endocrinology **139**, 1618-1629

40. Goodman, O. B., Krupnick, J. G., Santini, F., *et al.* (1996) Nature **383**, 447-450

41. Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998) Mol. Cell **1**, 565-574

42. Liakopoulos, D., Busgen, T., Brychzy, A., Jentsch, S., and Pause, A. (1999) Proc. Natl. Acad. Sci. USA. **96**, 5510-5515

43. Mori, S., Kanaki, H., Tanaka, K., Morisaki, N., and Saito, Y. (1995) Biochem. Biophys. Res. Commun. **217**, 224-229

44. Harrison, S. M., Barnard, R., Ho, K. Y., Rajkovic, I., and Waters, M. J. (1995) Endocrinology **136**, 651-659

45. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) Genes Dev. **12**, 3663-3674

46. Miyake, S., Lupher, M. L., Druker, B., and Band, H. (1998) Proc. Natl. Acad. Sci. USA. **95**, 7927-7932

47. Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) EMBO J. **16**, 6325-6336

48. Hou, D., Cenciarelli, C., Jensen, J. P., Nguyen, H. B., and Weissman, A. M. (1994) J. Biol. Chem. **269**, 14244-14247

49. Miyazawa, K., Toyama, K., Gotoh, A., Hendrie, P. C., Mantel, C., and Broxmeyer, H.E. (1994) Blood **83**, 137-145

50. Galcheva Gargova, Z., Theroux, S. J., and Davis, R. J. (1995) Oncogene **11**, 2649-2655

51. Cahoreau, C., Garnier, L., Djiane, J., *et al.* (1994) FEBS Lett. **350**, 230-234

Chapter IV

PROTEASOME INHIBITORS BLOCK A LATE STEP IN LYSOSOMAL TRANSPORT OF SELECTED MEMBRANE BUT NOT SOLUBLE PROTEINS

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ABSTRACT

The ubiquitin-proteasome pathway acts as a regulator of the endocytosis of selected membrane proteins. Recent evidence suggests that it may also function in the intracellular trafficking of membrane proteins. In this study, several models were used to address the role of the ubiquitin-proteasome pathway in sorting of internalized proteins to the lysosome. We found that lysosomal degradation of ligands, which remain bound to their receptors within the endocytic pathway, is blocked in the presence of specific proteasome inhibitors. In contrast, a ligand that dissociates from its receptor upon endosome acidification is degraded under the same conditions. Quantitative electron microscopy showed that neither the uptake nor the overall distribution of the endocytic marker bovine serum albumin-gold is substantially altered in the presence of a proteasome inhibitor. The data suggest that the ubiquitin-proteasome pathway is involved in an endosomal sorting step of selected membrane proteins to lysosomes, thereby providing a mechanism for regulated degradation.

Abbreviations used:

EGFR, epidermal growth factor receptor; GH, growth hormone; GHR, growth hormone receptor; LDL, low density lipoprotein; LRP, LDL receptor-related protein; MVB, multivesicular body; NGF, nerve growth factor; PDGFR, platelet-derived growth factor receptor; RAP, receptor-associated protein; Tf, transferrin; TrkA, receptor tyrosine kinase activated by NGF; UbE, ubiquitin-dependent endocytosis.

INTRODUCTION

After internalization from the plasma membrane, molecules are rapidly delivered to early endosomes, also known as sorting endosomes. Most of the soluble content of sorting endosomes is delivered to lysosomes for degradation, whereas the majority of membrane-bound proteins recycle back to the plasma membrane. Recycling receptors such as the transferrin receptor and the low-density lipoprotein (LDL) receptor are segregated into tubular membrane extensions of the sorting endosome and recycle with >99% efficiency, thereby avoiding proteolysis (reviewed in Mellman, 1996; Trowbridge *et al*., 1993). Recycling receptors are reused many times and are important for nutrient delivery and scavenging of non-functional proteins such as protease/protease inhibitor complexes and altered glycoproteins. On the other hand, signal-transducing membrane receptors such as the epidermal growth factor receptor (EGFR) and the growth hormone receptor (GHR) are transported together with their ligand into lysosomes for degradation, a process often referred to as signal down-regulation. Membrane proteins destined for lysosomal degradation are segregated into intraendosomal vesicles, which results in the formation of late endosomes or multivesicular bodies (MVBs), thus providing a mechanism to remove this class of proteins from the limiting membrane of the endosome (Felder *et al*., 1990). Down-regulation of growth factor receptors is important for cellular regulation; disrupted internalization or degradation often results in the loss of cell growth control (reviewed in Lemmon and Traub, 2000).

The ubiquitin-proteasome pathway controls a multitude of regulatory processes via ubiquitin-mediated degradation of essential cytosolic and nuclear proteins. The pathway comprises ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-ligase (E3), and a multisubunit protease, the 26S proteasome. The enzymes E1, E2, and E3 act in concert and accomplish the covalent attachment of multiple ubiquitin molecules to the specific target proteins. Polyubiquitinated proteins are then degraded by the 26S proteasome (reviewed in Hershko and Ciechanover, 1998). In addition to this well-known role in recognition by the proteasome, ubiquitination is also involved in endocytosis and down-regulation of membrane receptors, transporters and channels (reviewed in Hicke, 1999; Strous and Govers, 1999). In yeast it was shown that a single ubiquitin moiety is sufficient to mediate internalization of an activated receptor that lacks all cytoplasmic tail sequences (Roth and Davis, 2000; Shih *et al*., 2000). The uracil permease Fur4p undergoes ubiquitin-dependent internalization and vacuolar degradation with lysine-63 in ubiquitin serving as a critical residue for ubiquitin chain addition (Galan and Haguenauer-Tsapis, 1997). Recent evidence suggests that the ubiquitin-proteasome pathway may also regulate protein sorting after the initial internalization step, at the level of the endosome. The tyrosine kinase adaptor protein c-Cbl mediates EGFR ubiquitination and its subsequent lysosomal and/or proteasomal degradation. c-Cbl does not accelerate internalization of the EGFR but may

function at the endosome to facilitate sorting of the receptor into the MVB, thereby attenuating kinase signaling (Levkowitz *et al*., 1998). In yeast, the F-box protein Rcy1p is involved in endocytic membrane traffic and recycling out of an early endosome. Members of the F-box family of proteins have been shown to mediate ubiquitination of substrate proteins as components of SKP1/cullin/F-box ubiquitin ligase complexes (reviewed in Deshaies, 1999). Degradation of the α-factor receptor and uracil permease is inhibited at a postinternalization step in *Rcy1Δ* mutant cells (Wiederkehr *et al*., 2000).

The GHR is a mammalian plasma membrane protein whose internalization is mediated by the ubiquitin-proteasome pathway (Strous *et al*., 1996). A 10 amino acid motif within the GHR cytosolic tail (the UbE motif; DSWVEFIELD) is involved in both receptor ubiquitination and endocytosis (Govers *et al*., 1999). Mutation of residue Phe-327 within this motif to alanine, abolished receptor ubiquitination and ligand internalization and degradation (Govers *et al*., 1997). GHR ubiquitination occurs at the cell surface and coincides with the recruitment of the receptor to clathrin-coated membrane areas (van Kerkhof *et al*., 2001). Growth hormone (GH)-induced internalization of the full-length GHR is inhibited in the presence of specific proteasome inhibitors, whereas a receptor truncated at position 369 enters the cells unaffected (van Kerkhof *et al*., 2000). In addition to the ubiquitin-dependent endocytosis signal, the cytosolic tail of the GHR contains a di-leucine motif. On truncation of the GHR at amino acid residue 349, this di-leucine motif becomes functional and mediates ubiquitin system-independent internalization (Govers *et al*., 1998). We used this feature to study the involvement of the ubiquitin-proteasome pathway in lysosomal targeting. For the GHR truncated at amino acid 349, both the UbE-motif and proteasomal activity are required for endosomal sorting of the GH--GHR complex to the lysosome. In addition, we show that proteasome inhibitors block the degradation of nerve growth factor (NGF), the ligand for the receptor tyrosine kinase TrkA, at a postinternalization step. Transport of the general endocytic marker, bovine serum albumin (BSA)-gold, and acid-labile ligands was not blocked under these conditions. Together, these data reveal an important role for the ubiquitin-proteasome pathway in endosomal sorting of membrane proteins for lysosomal degradation.

MATERIALS AND METHODS

Materials and Antibodies

The polyclonal antibody generated against amino acid residues 271-318 of the cytosolic tail of the GHR (anti-T) was described previously (van Kerkhof *et al*., 2000). Human GH was a gift of Eli Lilly (Indianapolis, IN). MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) and *clasto*-lactacystin β-lactone were purchased from Calbiochem-Novabiochem (San Diego, CA) and transferrin (Tf) was purchased from Sigma (St. Louis, MO). Human recombinant RAP was expressed in a glutathione

(GST) expression vector and isolated as described previously (Li *et al*., 2000). Murine NGF (2.5S) was obtained from Promega (Leiden, The Netherlands).

Plasmids, Cell Culture, and Transfection

Full-length rabbit GHR cDNA in pCB6 was described (Strous *et al*., 1996). The truncated GHR cDNAs GHR(349) and GHR(399) were subcloned into the CMV-NEO expression plasmid pcDNA3.1 (Invitrogen, Groningen, The Netherlands) as previously described (Govers *et al*., 1998). cDNA of mutants GHR(349)(F327A) and GHR(399)(K271-362R) were constructed as previously described (Govers *et al*., 1999). Rat TrkA cDNA was kindly provided by Dr. D. Holtzman (Washington University School of Medicine, St. Louis, MO) and subcloned from pDM115 into the CMV-NEO expression plasmid pcDNA3.1. The construction of mLRP4T100, the membrane-containing minireceptor of LDL receptor-related protein (LRP) was previously described (Li *et al.*, 2000). The Chinese hamster cell line ts20, bearing a thermolabile ubiquitin-activating enzyme E1, was used in this study (Kulka *et al*., 1988). cDNA constructs were transfected into the ts20 cells with the use of the calcium phosphate transfection procedure. For all constructs stably expressing clonal cell lines were obtained. The ts20 cells were grown at 30°C in minimum essential medium α (MEMα) supplemented with 10% fetal calf serum, 4.5 g/l glucose, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.45 mg/ml geneticin. For experiments cells were grown in 60-mm dishes in the absence of geneticin to a confluence of ~75% and 10 mM sodium butyrate was added overnight to increase GHR expression (Strous *et al*., 1996). The LRP-null Chinese hamster ovary (CHO) cell line stably transfected with the mLRP4T100 cDNA was cultured at 37°C in Ham's F12 medium (Li *et al*., 2000).

Ligand Binding, Internalization, and Degradation

^{125}I-human GH and ^{125}I-RAP were prepared with the use of chloramine T (Strous *et al*., 1996). ^{125}I-NGF was prepared with the use of lactoperoxidase (Sutter *et al*., 1979). For internalization studies, cells were grown in 12-well plates, washed with MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA, and incubated in a waterbath. ^{125}I-GH (8 nM) or ^{125}I-NGF (1 nM) was bound on ice for 2 h, the cells were washed free of unbound ligand, and incubated for 0-30 minutes at 30°C. Membrane-associated ligand was removed by acid wash (0.15 M NaCl, 50 mM glycine, 0.1% BSA pH 2.5) on ice. Internalized ligand was determined by measuring the radioactivity after solubilization of the acid-treated cells in 1 N NaOH with the use of a LKB gamma counter. For degradation studies, cells were incubated with ^{125}I-GH (8 nM) or ^{125}I-RAP (5 nM) for 6 minutes at 30°C. The medium was aspirated and the cells were washed and incubated in medium without ligand. At the indicated times, the medium was collected and precipitated with 1 volume of ice-cold 20% trichloroacetic acid (TCA) for 30 min on ice. Acid-soluble radioactivity was determined in the supernatant after centrifugation and was used as a measurement for degraded ligand. Membrane associated and internalized ligands were determined as described above. Nonspecific degradation was determined in the presence of excess unlabeled ligand and subtracted.

Transferrin Recycling

Tf was saturated with Fe^{3+} and labeled with ^{125}I with the use of iodo-beads (Pierce, Rockford, IL) according to standard procedures. The ts20 cells were grown in 6-cm dishes and depleted from serum by 60-min incubation in MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1 % BSA at 30°C. ^{125}I-Tf was added at 2 μg/ml and cells were incubated in the presence of ligand for 30 min. The medium was aspirated and cells were washed on ice for 5 min with buffer pH 5 [20 mM 2-(N-morpholino)ethanesulfonic acid pH 5.0, 130 mM NaCl, 50 μM desferal, 2 mM $CaCl_2$, 0.1% BSA] followed by 10 min on ice with buffer pH 7.4 (MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA).

Then cells were incubated in MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA containing 50 μM desferal at 30°C. At the indicated time points, 200 μl samples were taken and the amount of released ^{125}I-Tf was measured with the use of a LKB gamma counter. Background label was determined in the presence of 200 μg/ml of unlabeled Tf and subtracted. TCA-precipitation of the medium verified that the released ^{125}I-Tf was not degraded.

Metabolic Labeling

Cells were grown in 60-mm dishes and incubated in methionine- and cysteine-free MEM. Then [^{35}S]methionine (3.7 MBq/ml Tran^{35}S Label, 40 Tbq/mmol, ICN, Costa Mesa, CA) was added and the incubation was continued at 30°C in a CO_2 incubator. The radioactivity was replaced with medium containing 100 μM unlabeled methionine, 0.1% BSA and 16 nM GH and chased for 0-60 min. Cells were lysed and samples were immunoprecipitated (see below). Radioactivity was determined with the use of a Storm imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image QuaNT software, version 4.2a.

Cell Lysis and Immunoprecipitation

Immunoprecipitations were performed as described previously (Strous *et al*., 1996). For GHR immunoprecipitations, cells were lysed on ice in 0.3 ml of lysis buffer containing 1% TritonX-100, 1 mM EDTA in phosphate-buffered saline (PBS), containing 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μM MG-132, and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation of the supernatant was carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% BSA in PBS with the various inhibitors. The lysates were incubated with the indicated antibodies for 2 h on ice and immune complexes were isolated using protein A-agarose beads (Repligen, Cambridge, MA). The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. Immune complexes were subjected to SDS-PAGE and immunoblotting as described (Govers *et al*., 1997).

Electron Microscopy

BSA was coupled to 5-nm gold particles and dialyzed overnight against PBS at 4°C. Cells were incubated for 1 h in MEMα + 0.1% BSA in the presence or absence of 20 μM MG-132. After addition of BSA-gold at a final optical density of 5 at 520 nm, cells were further incubated for 1 h to label the entire endocytic pathway. After washing, cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 30 min on ice, followed by 3 h at room temperature. Further processing for ultrathin-cryosectioning was done as described previously (Slot *et al*., 1991). To pick up ultrathin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used (Liou *et al*., 1996).

Semiquantitative Analysis of BSA-Gold Distribution

To establish the distribution of internalized BSA-gold, per condition 50 cell profiles with a visible nucleus were analyzed at magnification of 25.000x. The number of gold particles located over a specific compartment was expressed as a percentage of total gold. The various endocytic compartments were distinguished by morphological criteria, which were deduced as a general concept from studies on the endocytic pathway of a large variety of cells (De Wit *et al*., 1999; Kleijmeer *et al*., 1997; Klumperman *et al*., 1991 and 1993). Primary endocytic vesicles and tubules were recognized by size (80-90 nm) and electron lucent lumen. Recycling vesicles and tubules had an electron dense lumen and were 60 nm in diameter. Early or sorting endosomes were recognized as elongated, irregular-shaped vacuoles with an electron lucent content and few internal vesicles. Late endosomes or MVBs

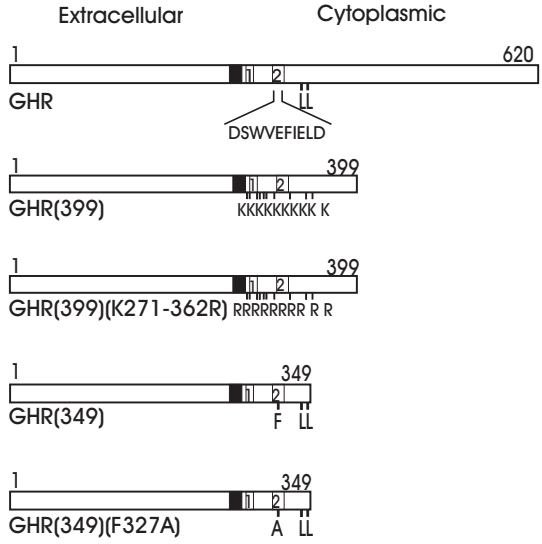
were characterized by their numerous internal vesicles, whereas lysosomes were defined by their electron dense content with the occasional appearance of membrane sheets. Clathrin-coated pits were defined as invaginations of the plasma membrane positive for clathrin, which was recognized by its electron dense appearance. Clathrin-coated vesicles near the plasma membrane were counted as a separate category, but part of these might in fact be connected to the plasma membrane, out of the plane of sectioning. Noncoated, flask-like, and sometimes branched invaginations were designated as caveolae. Membranes located in the vicinity of the *trans*-side of a Golgi stack were assigned as *trans*-Golgi area.

RESULTS

GHR Amino Acid Phenylalanine 327 Is Required for Both Receptor and Ligand Degradation

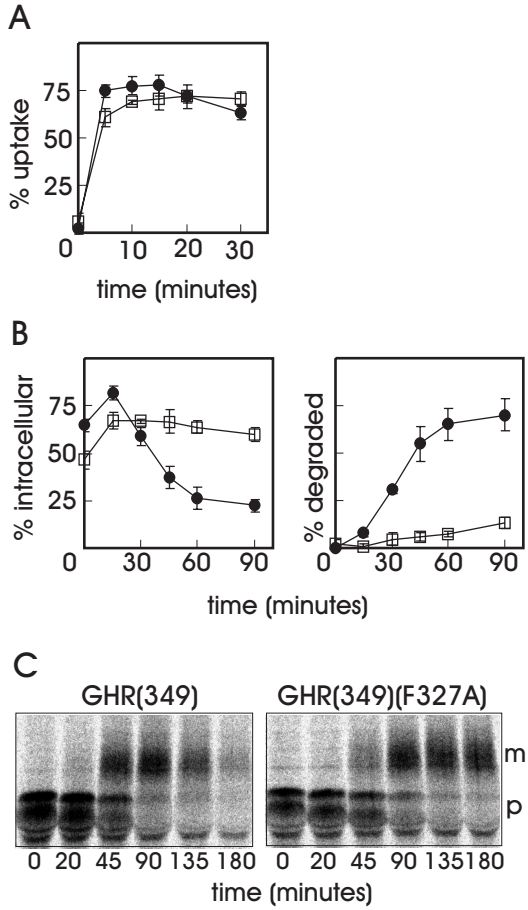
GHR endocytosis is mediated by the ubiquitin-proteasome pathway via a 10 amino acid internalization motif (UbE motif; DSWVEFIELD) (Govers *et al*., 1999; Strous *et al*., 1996). Previously, we identified a di-leucine motif in the cytosolic tail of the receptor, which upon truncation at amino acid 349 [GHR(349), Figure 1], is activated and mediates internalization in an ubiquitin system-independent fashion (Govers *et al*., 1998). Mutation of phenylalanine residue 327 to alanine in the UbE motif abolished ubiquitination of both full-length and truncated GHR (Govers *et al*., 1999) but did not influence the internalization of the truncated GHR(349) (Figure 2A). To determine whether the truncated receptor can direct its ligand to the degradation pathway, cells were incubated with ^{125}I-GH and chased for various time points in the absence of ligand after which the amount of TCA soluble radioactivity in the medium was analyzed, indicating ^{125}I-GH degradation. In the GHR(349) transfected cells, 50-75% of the lig-

Figure 1. Schematic representation of the wild-type and mutant GHRs



For the GHR mutants, truncated at residue 399, all cytoplasmic lysines and lysine mutations are indicated. For GHR mutants truncated at residue 349, Phe327 or Ala327 and Leu347, Leu348 are indicated. The black square represents the transmembrane domain. The numbered boxes represent box 1 and box 2, corresponding to the conserved homology domains within members of the cytokine receptor family. DSWVEFIELD indicates the position of the UbE motif, which is important for ubiquitination and endocytosis.

Figure 2. Phe327 is required for degradation of the truncated GHR(349)

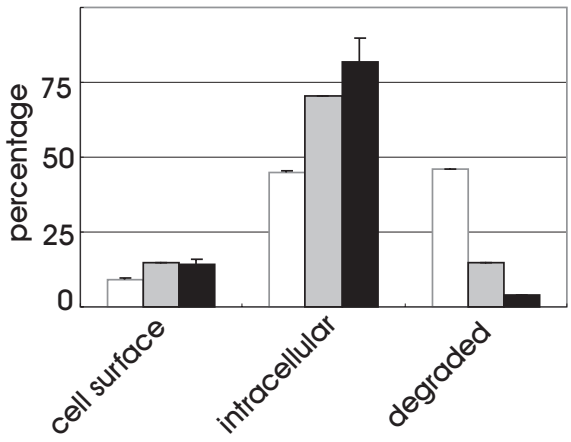


(A) GHR(349) or GHR(349)(F327A) expressing ts20 cells were incubated for 2 h on ice with 8 nM ^{125}I-GH. Unbound label was removed and the cells were incubated at 30°C for the indicated times. The amounts of internalized ^{125}I-GH are plotted as a percentage of the cell-associated radioactivity after binding on ice. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD.
●, GHR(349); □, GHR(349)(F327A).
(B) Cells were incubated for 6 min at 30°C with 8 nM ^{125}I-GH. The ligand was removed and the cells were incubated for the indicated times at 30°C. At each time point the amount of cell surface, internalized, and degraded ligand was determined as explained in MATERIALS AND METHODS. The amount of ^{125}I-GH is plotted as a percentage of total radioactivity. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD. (Left) Intracellular ^{125}I-GH. (Right) Degraded ^{125}I-GH in the medium.
●, GHR(349); □, GHR(349)(F327A).
(C) Cells were labeled with [^{35}S]methionine for 20 min at 30°C and chased in the absence of radioactivity for the indicated times. GHR was immunoprecipitated with anti-GHR antibody (anti-T). p, precursor GHR (50 kDa); m, mature GHR (70 kDa).

and was found intracellularly within the first 15 min (Figure 2B, left). Thereafter, the amount of intracellular ligand decreased rapidly with a concomitant increase of degraded ligand as acid-soluble radioactivity in the medium (Figure 2B, right). Endocytosis of ligand by the GHR(349)(F327A) mutant (Figure 1) was comparable to GHR(349) during the first 15 min. However, upon prolonged chase times, only a minor decrease in intracellular ligand was measured and almost no degraded ligand was detected in the medium, which is in striking contrast to what was observed with GHR(349). Thus, mutation of phenylalanine 327 in the UbE motif of the truncated GHR(349) has no effect on GH internalization but interferes significantly with its degradation.

Next, we analyzed the turnover of the truncated receptor itself with the use of pulse-chase labeling with [^{35}S] methionine. The receptor is synthesized as a glycoprotein precursor (Figure 2C, p) and converted in the Golgi apparatus to the complex glycosylated mature form (Figure 2C, m). For the truncated GHR(349) we observed a rapid dis-

Figure 3. Effect of proteasome inhibitors on GH degradation



GHR(349) expressing ts20 cells were incubated with 20 μM *clasto*-lactacystin β-lactone (2 h), 20 μM MG-132 (1 h) or solvent alone (control), then 8 nM ^{125}I-GH was added and the incubation was continued for 6 min at 30°C. The ligand was removed and the cells were incubated for 45 min at 30°C. The amount of cell surface, internalized, and degraded ligand was determined as explained in MATERIALS AND METHODS. The amount of ^{125}I-GH is plotted as a percentage of total radioactivity. Each bar represents the mean value of one experiment performed in duplicate ± SD. □, Control; ▒, β-Lactone; ■, MG-132.

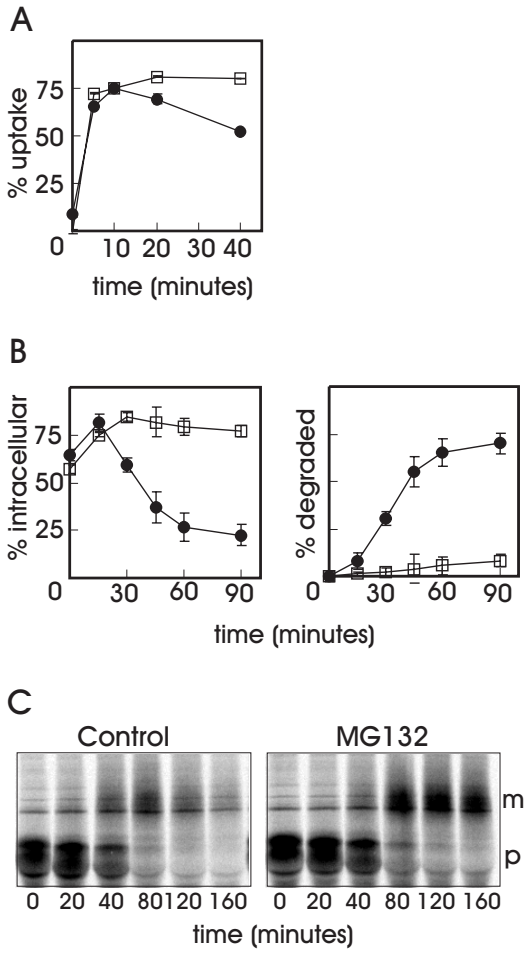
appearance of the mature form after prolonged chase times in the presence of GH, indicating a fast degradation of the receptor. Although the maturation of the mutated GHR(349)(F327A) was somewhat delayed as compared to GHR(349), quantification showed that the half-life of the mature mutant receptor was approximately fivefold prolonged. Together, these results show that residue phenylalanine 327 in the truncated GHR(349) is required for efficient degradation of both ligand and receptor. As the degradation of the ligand occurs in lysosomes (Murphy and Lazarus, 1984; Yamada *et al.*, 1987), we conclude that the GHR UbE motif is required for endosome-to-lysosome sorting of both receptor and ligand.

Proteasomal Activity Is Required for Degradation of a Truncated GHR and Its Ligand

Because the UbE-motif is involved in GHR ubiquitination, we investigated the role of the ubiquitin-proteasome pathway in the degradation of GH, with the use of proteasome inhibitors. The peptide aldehyde MG-132 is a substrate analogue and a reversible inhibitor of the chymotrypsin-like activity of the proteasome. Because peptide aldehydes might inhibit certain lysosomal cysteine proteases and the calpains, it is important to show that similar biological effects occur with other proteasome inhibitors. Lactacystin and its derivative *clasto*-lactacystin β-lactone are structurally different from the peptide aldehyde and act as a pseudo substrate that becomes irreversibly linked to the active site threonine of the proteasome β-subunits (Lee and Goldberg, 1998; Rock *et al*., 1994). Lactacystin shows high specificity for the proteasome but can also inhib-

it cathepsin A (Ostrowska *et al.*, 1997). GHR(349)-transfected cells were used to compare the effect of two specific proteasome inhibitors, MG-132 and *clasto*-lactacystin β-lactone (Craiu *et al*., 1997) on ^{125}I-GH degradation. After a short incubation with ^{125}I-GH, cells were chased for 45 min, whereafter the amounts of intracellular and degraded ligand in the medium were determined (Figure 3). In untreated cells (Control), 45% of the ligand was found intracellular and about the same amount was degraded. In cells treated with either of the proteasome inhibitors, the amount of intracellular GH was markedly increased compared with the control cells, however, degradation was almost completely inhibited. Because the two proteasome inhibitors showed a similar effect on ligand internalization and degradation, a more detailed analysis was performed with

Figure 4. MG-132 inhibits the degradation of the truncated GHR(349) and its ligand



(A) GHR(349) expressing ts20 cells were incubated for 1 h with 20 μM MG-132 or solvent and put on ice for 2 h with 8 nM ^{125}I-GH. Unbound label was removed and the cells were incubated at 30°C in the absence or presence of MG-132 as indicated. The amount of internalized ^{125}I-GH is plotted as a percentage of the cell-associated radioactivity after the 2-h binding on ice. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD. ●, control; □, MG-132.

(B) GHR(349) expressing ts20 cells were incubated for 1 h at 30°C with 20 μM MG-132 or solvent (control), then 8 nM ^{125}I-GH was added and the incubation was continued for 6 min. The ligand was removed and the cells were incubated at 30°C as indicated. At each time point the amount of cell surface, internalized, and degraded ligand was determined as explained in MATERIALS AND METHODS. The amount of ^{125}I-GH is plotted as a percentage of total radioactivity. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD. (Left) Intracellular ^{125}I-GH. (Right) Degraded ^{125}I-GH in the medium. ●, control; □, MG-132.

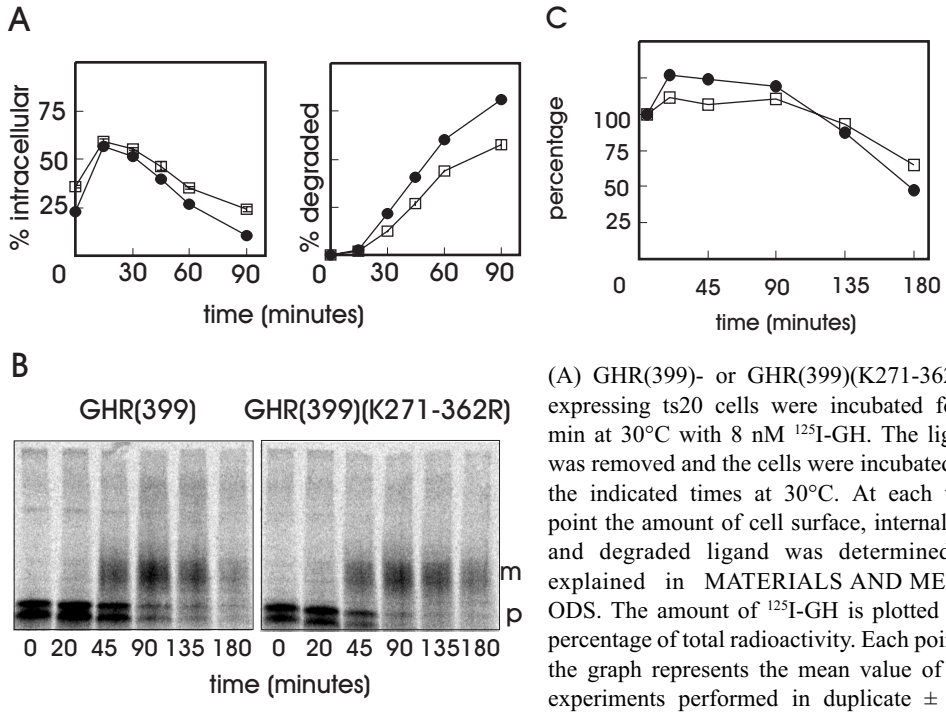
(C) GHR(349 expressing ts20 cells were labeled with [^{35}S]methionine for 20 min after preincubation for 1 h with or without 20 μM MG-132. Cells were chased in the absence of radioactivity for the indicated times. GHR was immunoprecipitated with anti-GHR antibody (anti-T). p, precursor GHR (50 kDa); m, mature GHR (70 kDa).

only MG-132 (Figure 4). In Figure 4A the effect of MG-132 on the uptake of ^{125}I-GH after binding on ice was determined. Both in control and MG-132-treated GHR(349) cells, ~ 75% of the ligand was internalized after 10 min, indicating that the proteasome inhibitor has no effect on endocytosis. For the control cells it has been shown (Figure 2) that internalized GH is rapidly degraded, starting after ~15 minutes of chase. However, in the presence of proteasome inhibitor, the degradation of GH was almost completely inhibited (Figure 4B). To monitor the effect of the proteasome inhibitor on the fate of the truncated receptor, a pulse-chase labeling experiment with [^{35}S]methionine was performed. As seen in Figure 4C, the mature form of the receptor was rapidly degraded upon prolonged chase times. In the presence of MG-132, the maturation of the truncated GHR(349) was unaffected while degradation was almost completely inhibited, which is in line with the effect of the proteasome inhibitor on the degradation of GH. These results clearly indicate an involvement of the ubiquitin-proteasome pathway in the degradation of the truncated GHR(349) and its ligand at a postinternalization step.

Lysine Residues in Cytoplasmic Domain of GHR Are Not Required for Degradation

The preceding experiments indicate that the ubiquitin-proteasome pathway is involved in directing the GHR and its ligand to the degradative pathway. Next, we addressed the question whether ubiquitination of the receptor itself is required for this sorting step. Previously, with the use of a GHR truncated at amino acid 399 in which all the 10 cytoplasmic lysine residues were mutated to arginine [GHR(399)(K271-362R); Figure 1], we have shown that GHR ubiquitination is not required for internalization at the plasma membrane. By replacing phenylalanine 327 for alanine in this mutant, we could show that the internalization is controlled by the UbE motif (Govers *et al*., 1999). Here, we have used cell lines stably expressing GHR(399) or truncation mutant GHR(399)(K271-362R) to measure the degradation of ^{125}I-GH. Both cell lines showed comparable amounts of intracellular ligand after incubation with ^{125}I-GH, again indicating that there is no effect on internalization if the GHR tail lacks attachment sites for ubiquitin (Figure 5A, left). After prolonged incubation, the percentage of intracellular ligand decreased, due to the degradation of the ^{125}I-GH, as can be seen in the Figure 5A, right. Degradation of GH in the case of the lysine-less GHR was only slightly less efficient compared with the lysine-containing truncation, strongly indicating that ubiquitination of the GHR itself is not required for its degradation. The degradation of the truncated receptors was monitored with the use of a pulse-chase labeling with [^{35}S]methionine (Figure 5B). As shown by immunoprecipitation of the GHR, the signal for the mature form decreased rapidly upon prolonged chase times, for both GHR(399) and GHR(399)(K271-362R) (Figure 5C). From these experiments we conclude that ubiquitination of the GHR itself is not required for the ubiquitin-proteasome pathway-dependent sorting to the degradative pathway.

Figure 5. Degradation of GHR and ligand is independent of lysine residues

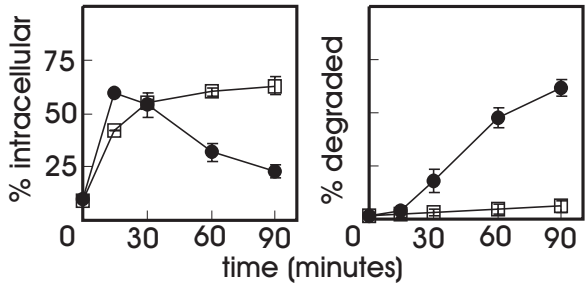


(A) GHR(399)- or GHR(399)(K271-362R)-expressing ts20 cells were incubated for 6 min at 30°C with 8 nM ^{125}I-GH. The ligand was removed and the cells were incubated for the indicated times at 30°C. At each time point the amount of cell surface, internalized and degraded ligand was determined as explained in MATERIALS AND METHODS. The amount of ^{125}I-GH is plotted as a percentage of total radioactivity. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD. (Left) Intracellular ^{125}I-GH. (Right) Degraded ^{125}I-GH in the medium. ●, GHR(399); □ GHR(399)(K271-362R). (B) Cells were labeled with [^{35}S]methionine for 20 min at 30°C and chased in the absence of radioactivity for the indicated times. GHR was immunoprecipitated with anti-GHR antibody (anti-T). p, precursor GHR (60 kDa); m, mature GHR (80 kDa). (C) The amounts of radioactivity of the total lanes were determined with the use of Image Quant software and were expressed as percentages of the radioactivity incorporated in the lanes at 0 min. ●, GHR(399); □, GHR(399)(K271-362R).

Proteasome Inhibitors Inhibit Degradation of TrkA-bound NGF at Level of Endosomes

Internalization and degradation of the GHR depends on the UbE motif in its cytoplasmic tail. Next, we addressed the question whether the degradation of a receptor, which is sorted into the degradative pathway but does not contain an obvious UbE motif, is also regulated by the ubiquitin-proteasome pathway. Similar to other receptor tyrosine kinases, TrkA, the receptor tyrosine kinase for the neurothrophin NGF, dimerizes upon ligand binding, which in turn results in an activation of the intracellular kinase domain and rapid internalization (Grimes *et al.*, 1997). With the use of Chinese hamster ts20 cells stably transfected with TrkA we found that the ubiquitin-proteasome pathway is not involved in the endocytosis of this receptor (Alves dos Santos and Strous, unpublished results). We used these cells to determine the effect of proteasome inhibitors on the degradation of NGF. As seen in Figure 6, left, the internalization of the

Figure 6. Effect of MG-132 on degradation of ^{125}I-NGF



TrkA-expressing ts20 cells were incubated for 2 h with 20 μM MG-132 or solvent and put on ice for 1 h with 1 nM ^{125}I-NGF. Unbound label was removed and the cells were incubated at 30°C in the absence or presence of MG-132 as indicated. The amounts of ^{125}I-NGF are plotted as a percentage of the cell-associated radioactivity after 2-h binding on ice. (Left) Intracellular NGF. (Right) Degraded NGF. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD. ●, control; □, MG-132.

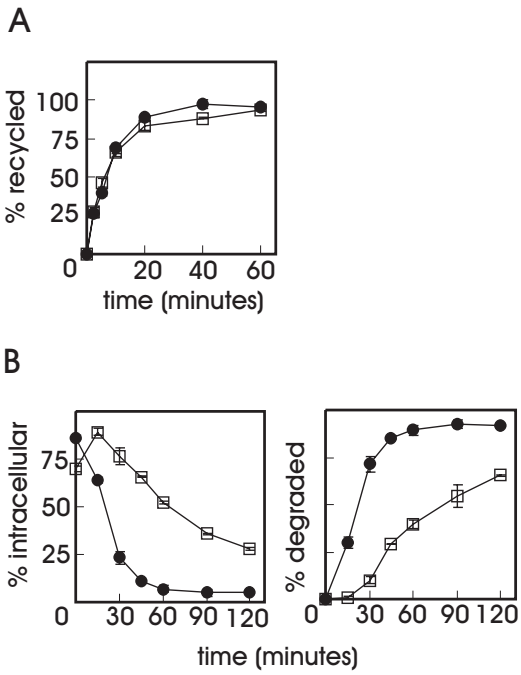
ligand was not inhibited in the presence of MG-132. After prolonged incubation, the intracellular amount of ^{125}I-NGF decreased in the control cells and could be detected as TCA-soluble radioactivity in the medium (Figure 6, right). In the MG-132 treated cells, the label remained intracellular during the chase with no measurable degradation of NGF. This result indicates an involvement of the ubiquitin-proteasome pathway in lysosomal transport of NGF bound to TrkA.

Dissociating Ligands and an Endocytic Marker Are Transported to Lysosomes in Presence of Proteasome Inhibitors

Most of the soluble content of sorting endosomes is delivered to lysosomes for degradation, while the majority of membrane proteins recycle from the endocytic pathway back to the plasma membrane. To address the question whether proteasome inhibitors interfere with general mechanisms in membrane transport, we examined their effect on the recycling pathway and on the degradation of a ligand that dissociates from its receptor upon endosome acidification. To this end, ts20 cells were loaded with ^{125}I-Tf in the absence or presence of MG-132 after which the amount of recycling was measured by determining the release of internalized Tf into the medium. As can be seen in Figure 7A, recycling of Tf was unaffected in the presence of the proteasome inhibitor.

The LRP is an endocytic receptor that belongs to the LDL receptor gene family (Herz *et al*., 1988). Ligand interactions with LRP can be antagonized by a 39-kDa receptor-associated protein (RAP). The recombinant form of RAP has been used extensively in the study of ligand-receptor interactions. Endocytosis of cell-surface bound RAP is rapid and the internalized ligand is delivered to lysosomes while the receptor recycles to the cell surface (Czekay *et al*., 1997; Iadonato *et al*., 1993). In this study we used an LRP-null CHO cell line, stably transfected with the LRP minireceptor mLRP4T100, which mimics the function and trafficking of LRP (Li *et al*., 2000). Cells were incubated for a short time with ^{125}I-RAP in the presence or absence of MG-132. After prolonged incubation in the absence of labeled ligand, the amount of internalized

Figure 7. Effect of MG-132 on transferrin recycling and degradation of ^{125}I-RAP



(A) ts20 cells were serum depleted for 1 h with or without 20 μM MG-132 and then loaded with 2 μg/ml ^{125}I-Tf for 30 min at 30°C. Cells were chilled on ice, plasma membrane-bound ^{125}I-Tf was removed and the cells were incubated at 30°C in medium containing 50 μM desferal in the absence or presence of MG-132. The release of ^{125}I-Tf was determined and expressed as a percentage of the total amount of radioactivity loaded in the cells. ●, control; □, MG-132.

(B) LRP-null CHO cells stably transfected with mLRP4T100 were incubated for 1 h at 37°C with or without 20 μM MG-132 before 5 nM ^{125}I-RAP was added. The incubation was continued for 6 min, after which the unbound radioactivity was removed and the cells were incubated at 37°C in the absence of ligand with or without MG-132 for the time points indicated. At each time point the amount of cell surface, internalized, and degraded ligand was determined as explained in MATERIALS AND METHODS. The amount of ^{125}I-RAP is plotted as a percentage of total radioactivity. Each point in the graph represents the mean value of two experiments performed in duplicate ± SD. (Left) Intracellular ^{125}I-RAP. (Right) Degraded ^{125}I-RAP in the medium. ●, control; □, MG-132.

and degraded ^{125}I-RAP was determined as described in MATERIALS AND METHODS (Figure 7B). As seen in the left panel, most of the ligand was already intracellular after 6 min of incubation, both in the absence or presence of MG-132. The amount of intracellular ligand decreased rapidly, accompanied by increased levels of TCA-soluble radioactivity in the medium (right). Degradation of RAP was less efficient in MG-132-treated cells, an effect that was also found in ts20 cells transfected with mLRP4T100 (van Kerkhof, unpublished results). MG-132 inhibited RAP degradation threefold, whereas both GH and NGF degradation was 16-17-fold decreased. Even although MG-132 affects RAP degradation, the effect differs in magnitude from the effect on GH and NGF degradation: once internalized, all RAP molecules are completely degraded, be it somewhat later (Figure 7B). This is not the case for NGF, neither for GH, if taken up via GHR(349)(F327A) or in the presence of MG-132: they are probably forced into the recycling pathway, escaping degradation. Additional evidence for this was provided by experiments with GHR(349)(F327A)-expressing cells, which were continuously incubated with ^{125}I-GH: cell-associated radioactivity increased linearly with the GHR rate of synthesis, wheras no soluble radioactivity was released from

the cells (van Kerkhof, unpublished results). Why MG-132 had a moderate affect on RAP degradation is unclear at the moment. One reason might be that the inhibitor slows down lysosomal degradation.

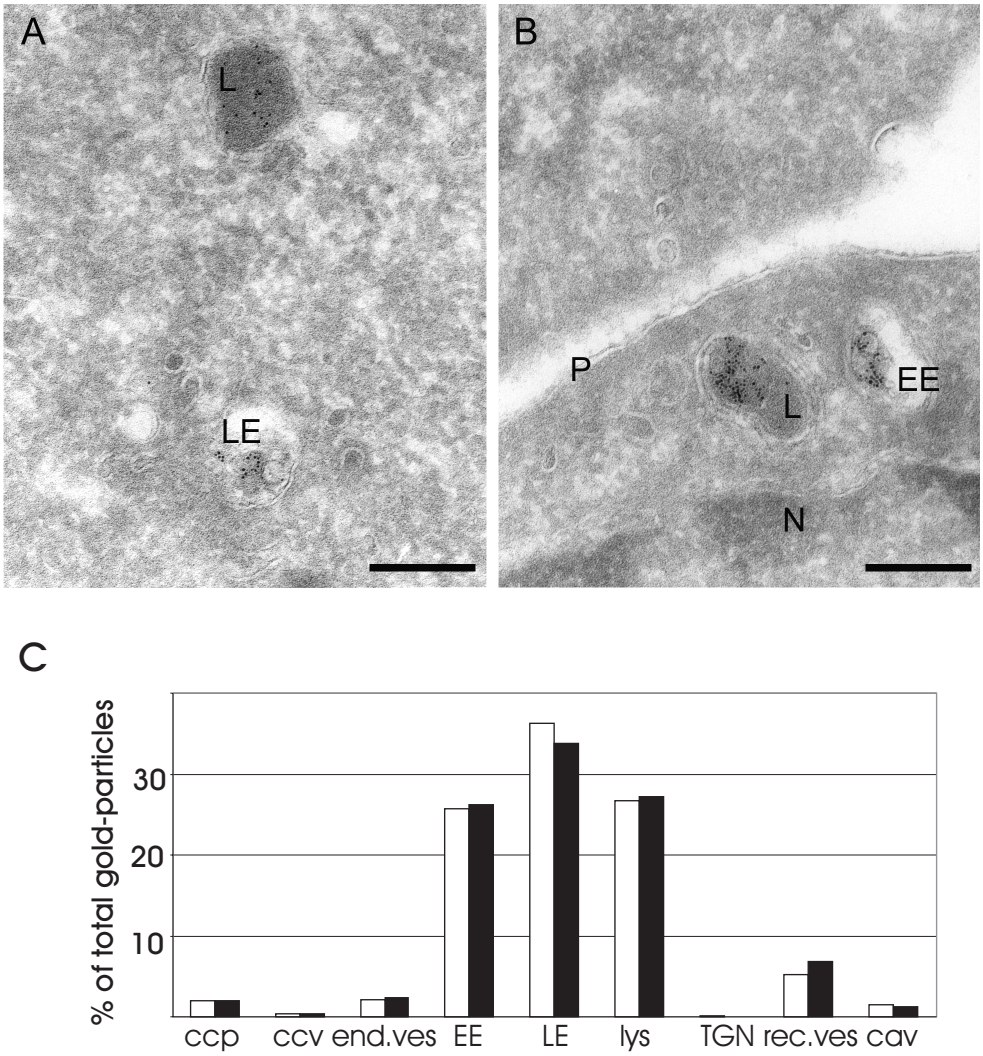
To investigate a possible general effect of proteasome inhibitors on the lysosomal pathway, we visualized transport along the endocytic tract with internalized BSA-gold (Slot *et al*., 1988). After 1-h uptake, significant amounts of BSA-gold reached the lysosomes in both control (Figure 8A) and MG-132-treated cells (Figure 8B). Quantitative analysis of the relative distribution of BSA-gold over the distinct endocytic compartments (for detailed definitions, see MATERIALS AND METHODS) revealed nearly identical distribution patterns (Figure 8C). Of particular interest in this respect, in control cells 36 and 26% of total gold was found in late endosomes and lysosomes, respectively, whereas in MG-132-treated cells these figures amounted to 34 and 27%. These data are a strong indication that transport of BSA-gold to lysosomes is unaltered in the presence of proteasome inhibitors. The low amount of BSA-gold in TGN and recycling vesicles and tubules is in agreement with the notion that this marker mainly follows the degradative pathway. Together, the data demonstrate that transport of an endocytic marker is unaltered and that degradation of a dissociating ligand is only slightly inhibited after incubation with MG-132, and indicate that proteasome inhibitors block a late step in lysosomal transport of selected membrane proteins but not in transport of soluble proteins.

DISCUSSION

Accumulating evidence suggests a role for ubiquitination in regulating protein sorting in the endosomal system. In this study, we used several models to address the role of the ubiquitin-proteasome pathway in sorting of internalized proteins at the level of the endosome. First, degradation of GH bound to GHR, a receptor that is ubiquitinated dependent on its UbE motif, was compared with degradation of NGF bound to TrkA, a receptor without an UbE motif. Both receptors remain bound to their ligand in endosomes. Proteasome inhibitors completely blocked the degradation of GH and NGF. In contrast, degradation of RAP, which needs to dissociate from its recycling receptor for targeting to lysosomes, was only slightly inhibited in the presence of the proteasome inhibitor, and transport of an general endocytic marker to the lysosome was unaffected. Together, the data suggest that proteasome inhibitors block the sorting of a select set of membrane proteins to the degradative pathway without interfering with transport of soluble proteins.

The GHR UbE motif is important for ubiquitination of the full-length receptor and its truncations. The truncated GHR(349) is internalized from the plasma membrane independent of the ubiquitin system by a C-terminally located di-leucine motif. Here we corroborated the finding that mutation of residue phenylalanine 327 in the UbE motif

Figure 8. Transport of BSA-gold is not altered by a proteasome inhibitor



(A) and (B) Ultrathin cryosections of wild-type GHR-expressing ts20 cells, which were incubated for 1 h with BSA coupled to 5-nm gold. (A) In control cells, BSA-gold was found in the entire endocytic tract. Here, localization in a late endosome (LE) and a lysosome (L) is shown. (B) In the presence of MG-132 transport of BSA-gold in the endosomal-lysosomal pathway is not affected, as illustrated by the presence of BSA-gold in early endosomes (EE) as well as in lysosomes. N, nucleus; P, plasma membrane. Bars, 200nm. (C) Relative distribution of internalized BSA-gold particles over distinct endocytic compartments was established as explained in MATERIALS AND METHODS and expressed as percentage of the total number of gold-particles counted (3803 and 3580 in control and MG-132-treated cells respectively). ccp, clathrin-coated pits; ccv, clathrin-coated vesicles; end.ves, primary endocytic vesicles and tubules; EE, early endosome; LE, late endosome; Lys, lysosome; TGN, *trans*-Golgi network; rec ves, recycling vesicles; cav, caveolae. □, control; ■, MG-132.

of GHR(349) did not interfere with initial internalization from the plasma membrane and provide novel evidence that this mutation abolished subsequent degradation of both receptor and ligand. Therefore, we conclude that the UbE motif is not only involved in endocytosis of the GHR but also in its subsequent sorting to the lysosomes. The data also indicate that the di-leucine motif, which is involved in endocytosis of this GHR truncation, does not mediate the sorting to the degradative pathway. The finding that one motif can mediate different transport steps is in itself not new. For tyrosine-based motifs it has been reported, that they can function at different sorting steps (Peters *et al*., 1990; Prill *et al*., 1993) and also the di-leucine motif can act as an internalization motif and in lysosomal targeting (reviewed in Hunziker and Geuze, 1996). The role of the ubiquitin-proteasome pathway in intracellular sorting to the lysosome was corroborated by the use of specific proteasome inhibitors. Because the use of pharmacological inhibitors could be a potential problem with regard to specificity, we used two different inhibitors: β-lactone and MG-132; both were able to inhibit degradation of GH and GHR to the same extent (75-90%, Figure 3; Van Kerkhof *et al*., 2000). Although these inhibitors are structurally different, it cannot be excluded that part of the inhibition is due to an inhibitory effect on lysosomal hydrolases. The GH--GHR complex dissociates at a pH below 3, indicating that receptor and ligand remain associated in the endosomal system. A common localization throughout the endocytic pathway was shown for EGF and EGFR and similarly a pool of intact platelet-derived growth factor (PDGF)--PDGFR and NGF--TrkA receptor complexes could be detected in endosomes (Sorkin and Waters, 1993). We show that the degradation of NGF, internalized via its receptor, TrkA, is inhibited in the presence of proteasome inhibitors. Proteasome inhibitors were also shown to inhibit the degradation of the PDGFR (Mori *et al*., 1995) and the Met tyrosine kinase receptor (Jeffers *et al*., 1997). Furthermore, it was shown that proteasome inhibitors inhibit the down-regulation of the EGFR (Levkowitz *et al*., 1998) and the lysosomal degradation of interleukin-2 internalized by the interleukin-2 receptor (Yu and Malek, 2001), suggesting a possible role for the proteasome in regulating trafficking to the lysosome.

Do proteasome inhibitors interfere with trafficking to the lysosome or with the activity of lysosomal enzymes? Degradation of GH and NGF, which remain associated with their receptor, was inhibited 16-fold, whereas degradation of RAP, which dissociates from its receptor, was inhibited only threefold in the presence of MG-132. This difference in magnitude, together with the observation that mutation of the UbE motif causes comparable inhibition of GH degradation in the absence of MG-132, suggests a role of the ubiquitin-proteasome pathway in the delivery of GH and NGF to the lysosome. The most likely scenario is that inhibition of the ubiquitin-proteasome pathway induces recycling of the ligand--receptor complex: 1) More GH is associated with the cell surface after incubation with proteasome inhibitors (Figure 3). 2) Proteasome inhibitors do

not interfere with transferrin recycling (Figure 7A). 3) Morphological quantification data, with the use of the GHR(349)(F327A) mutant, showed co-localization of GH with transferrin receptor in recycling vesicles (Sachse and Klumperman, unpublished results). 4) Continuous uptake of ^{125}I-GH in this mutant resulted in accumulation of radioactivity, both intracellular and at the cell surface, suggesting recycling of the mutant receptor--ligand complex. Why is the degradation of RAP inhibited while the morphological data show that transport of the endocytic marker BSA-gold is unaltered in the presence of MG-132? One possible explanation is that the proteasome inhibitor inhibits lysosomal hydrolases to some extent which would result in a delayed degradation. Another possibility is that proteasome inhibitors interfere with the dissociation of receptor and ligand in the sorting endosome, resulting in recycling of RAP together with the receptor. This should result in more receptor and ligand at the cell surface, although the complex will be reinternalized very efficiently once it reaches the plasma membrane. Consistent with this, we detect more RAP (9 versus 4%) at the cell surface in the presence of proteasome inhibitors.

What is the molecular mechanism that regulates endosomal sorting to the lysosome? For a number of ligand-stimulated receptor tyrosine kinases, the tyrosine kinase adaptor c-Cbl is involved. Overexpression of c-Cbl increases ligand-induced ubiquitination and down-regulation of EGFR, PDGFR and colony stimulating factor-1 receptor (Lee *et al*., 1999; Levkowitz *et al*., 1998; Miyake *et al*., 1998). For the EGFR, down-regulation depends on its intrinsic kinase activity and involves the *trans*-phosphorylation of the c-Cbl adaptor. For the GHR, the mechanism is unknown but must be different, because although the receptor is tyrosine phosphorylated upon addition of GH, no phosphorylation of c-Cbl could be detected (van Kerkhof, unpublished results). Possibly, the GHR UbE motif may serve, directly or via adaptor proteins, as an anchoring site for the ubiquitinating enzymes leading to coated pit localization, internalization and subsequent endosomal sorting. It was recently suggested that the ubiquitination state of proteins at the late endosome might help concentrate them in regions that will invaginate and form the internal vesicles (Amerik *et al*., 2000). The results with the truncated GHR without intracellular attachment sites for ubiquitin [GHR(399)(K271-362R)] show that ubiquitination of the receptor itself is not required for lysosomal sorting. Which proteins do need to be ubiquitinated is unclear at present, possibly it is the receptor that brings the ubiquitination machinery in proximity to the sorting machinery that is regulated by ubiquitination. Dynamic ubiquitination/deubiquitination of components of the endocytic machinery could play a role in the subsequent transport steps along the endocytic pathway. Ligand-induced ubiquitination was shown for Eps15, a clathrin-coated pit-associated protein required for EGFR uptake (van Delft *et al*., 1997) and genetic data implicate the product of the *Drosophila liquid facets* gene, epsin, which binds to Eps15, as a target for the fat facets deubiquitinating enzyme (Cadavid *et*

al., 2000). From recent data it appeared that the yeast Doa4 deubiquitinating enzyme localizes reversibly with the late endosome/ prevacuolar compartment along with a group of proteins essential for targeting of membrane proteins to the vacuole and it was proposed that Doa4 is responsible for deubiquitination events at the late endosome (Amerik *et al*., 2000).

An important question that remains is what is the target protein for the proteasome? It is not clear whether the down-regulated receptors are degraded in lysosomes, or by the proteasome, or both. However, because GH and NGF are also not degraded in the presence of proteasome inhibitors, this would favor a model in which one of the components of the endocytic sorting machinery is the target for the proteasome. Inhibition of degradation of (part of) this component would then lead to inhibition of assembling the sorting machinery and possibly result in recycling of the receptor--ligand complex. In the case of GHR sorting, a direct role for both the ubiquitin system and the proteasome is anticipated, although the target of the proteasome is probably not the GHR cytosolic tail. It remains to be seen whether receptors, in which c-Cbl plays a role in lysosomal sorting, are direct targets of the proteasome. It is also possible that there is no direct role for the proteasome. Use of proteasome inhibitors bears the risk of exhausting the cells of free ubiquitin (Swaminathan *et al*., 1999) which might lead to reduced ubiquitination of the target protein and reduced endosomal sorting. Recently, ubiquitin was implicated in retrovirus assembly and budding, a process in which the host machinery for endocytosis or MVB formation could play a role (Patnaik *et al*., 2000; Schubert *et al*., 2000). The late domain in the retroviral Gag protein was shown to recruit ubiquitin ligases to the site of viral assembly and it was suggested that the engagement of the ubiquitin conjugation machinery plays a crucial role in the release of retroviruses. Proteasome inhibitors caused defects in virus budding, possibly through depletion of the pool of free ubiquitin, suggesting that there is no direct role for the proteasome.

In conclusion, the results of this study point to a specific role of the ubiquitin-proteasome pathway in the regulated sorting of specific sets of membrane proteins. Based on our observation that ubiquitination of the GHR itself is not required for this sorting, we speculate that a specific membrane protein recruits a ubiquitin ligase, which then directly or via ubiquitination of target proteins, recruits the sorting machinery to accomplish its subsequent degradation.

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REFERENCES

Amerik, A.Y., J. Nowak, S. Swaminathan, and M. Hochstrasser. 2000. Mol. Biol. Cell 11:3365-3380.

Cadavid, A.L., A. Ginzel, and J.A. Fischer. 2000. Development 127:1727-1736.

Craiu, A., M. Gaczynska, T. Akopian, *et al*. 1997. J. Biol. Chem. 272:13437-13445.

Czekay, R.P., R.A. Orlando, L. Woodward, *et al.* 1997. Mol. Biol. Cell 8:517-532.

Deshaies, R.J. 1999. Annu. Rev. Cell Dev. Biol. 15:435-467.

De Wit, H., Y. Lichtenstein, H.Y. Geuze, *et al*. 1999. Mol. Biol. Cell 10: 4163-4176.

Felder, S., K. Miller, G. Moehren, *et al*. 1990. Cell 61:623-634.

Galan, J.M., and R. Haguenauer-Tsapis. 1997. EMBO J. 16:5847-5854.

Govers, R., T. ten Broeke, P. van Kerkhof, A.L. Schwartz, and G.J. Strous. 1999. EMBO J. 18:28-36.

Govers, R., P. van Kerkhof, A.L. Schwartz, and G.J. Strous. 1997. EMBO J. 16:4851-4858.

Govers, R., P. van Kerkhof, A.L. Schwartz, and G.J. Strous. 1998. J. Biol. Chem. 273:16426-16433.

Grimes, M.L., E. Beattie, and W.C. Mobley. 1997. Proc. Natl. Acad. Sci. USA 94:9909-9914.

Hershko, A., and A. Ciechanover. 1998. Annu. Rev. Biochem. 67:425-479.

Herz, J., U. Hamann, S. Rogne, *et al*. 1988. EMBO J. 7:4119-4127.

Hicke, L. 1999. Trends Cell Biol. 9:107-112.

Hunziker, W., and H.J. Geuze. 1996. 18:379-389.

Iadonato, S.P., G. Bu, E.A. Maksymovitch, and A.L. Schwartz. 1993. Biochem. J. 296:867-875.

Jeffers, M., G.A. Taylor, K.M. Weidner, *et al*. 1997. Mol. Cell. Biol. 17:799-808.

Kleijmeer MJ, Morkowski S, Griffith JM, *et al.* 1997. J. Cell Biol. 139:639-649.

Klumperman J, Boekestijn JC, Mulder AM, *et al*. 1991. Eur. J. Cell Biol. 54:76-84.

Klumperman J, Hille A, Veenendaal T, *et al*. 1993. J. Cell Biol. 121:997-1010.

Kulka, R.G., B. Raboy, R. Schuster, *et al*. 1988. J. Biol. Chem. 263:15726-15731.

Lee, P.S., Y. Wang, M.G. Dominguez, *et al*. 1999. EMBO J. 18:3616-3628.

Lee, D.H., and A.L. Goldberg. 1998. Trends Cell Biol. 8:397-403.

Lemmon, S.K., and L.M. Traub. 2000. Curr. Opin. Cell Biol. 12:457-466.

Levkowitz, G., H. Waterman, E. Zamir, *et al*. 1998. Genes Dev. 12:3663-3674.

Li, Y., M. Paz Marzolo, P. van Kerkhof, *et al*. 2000. J. Biol. Chem. 275:17187-17194.

Liou, W., H.J. Geuze, and J.W. Slot. 1996. Histochem. Cell Biol. 106:41-58.

Mellman, I. 1996. Annu. Rev. Cell Dev. Biol. 12:575-625.

Miyake, S., M.L. Lupher, J., B. Druker, and H. Band. 1998. Proc. Natl. Acad. Sci. USA 95:7927-7932.

Mori, S., K. Tanaka, S. Omura, and Y. Saito. 1995. J. Biol. Chem. 270:29447-29452.

Murphy, L.J., and L. Lazarus. 1984. Endocrinology 115:1625-1632.

Ostrowska, H., C. Wojcik, S. Omura, *et al.* 1997. Biochem. Biophys. Res. Commun. 234:729-732.

Patnaik, A., V. Chau, and J.W. Wills. 2000. Proc. Nat. Acad. Sci. USA 97:13069-13074.

Peters, C., M. Braun, B. Weber, M. *et al*. 1990. EMBO J. 11:3497-3506.

Prill, V., L. Lehmann, K. von Figura, and C. Peters. 1993. EMBO J. 12:2181-2193.

Rock, K.L., C. Gramm, L. Rothstein, *et al.* 1994. Cell. 78:761-771.

Roth, A.F., and N.G. Davis. 2000. J. Biol. Chem. 275:8143-8153.

Schubert, U., D.E. Ott, E.N. Chertova, *et al*. 2000. Proc. Nat. Acad. Sci. USA 97:13057-13062.

Shih, S.C., K.E. Sloper-Mould, and L. Hicke. 2000. EMBO J. 19:187-198.

Slot, J.W., H.J. Geuze, S. Gigengack, G.E. Lienhard, and D.E. James. 1991. J. Cell Biol. 113:123-135.

Slot, J.W., H.J. Geuze, and A.H. Weerkamp. 1988. Methods Microbiol. 20:211-236.

Sorkin, A., and C.M. Waters. 1993. Bioessays 15:375-382.

Strous, G.J., and R. Govers. 1999. J. Cell Sci. 112:1417-1423.

Strous, G.J., P. van Kerkhof, R. Govers, *et al.* 1996. EMBO J. 15:3806-3812.

Sutter, A., R.J. Riopelle, R.M. Harris-Warrick, and E.M. Shooter. 1979. J. Biol. Chem. 254:5972-5982.

Swaminathan, S., A.Y. Amerik, and M. Hochstrasser. 1999. Mol. Biol. Cell 10:2583-2594.

Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993. Annu. Rev. Cell Biol. 9:129-161.

Van Kerkhof, P., R. Govers, C.Alves Dos Santos, *et al.* 2000. J. Biol. Chem. 275:1575-1580.

Van Kerkhof, P., M. Sachse, J. Klumperman, and G.J. Strous. 2001. J. Biol. Chem. 276:3778-3784.

Van Delft, S., R. Govers, G.J. Strous, *et al*. 1997. J. Biol. Chem. 272:14013-14016.

Wiederkehr, A., S. Avaro, C. Prescianottobaschong, *et al*. 2000. J. Cell Biol. 149:397-410.

Yamada, K., K.E. Lipson, and D.B. Donner. 1987. Biochemistry 26:4438-4443.

Yu, A., and T. Malek. 2001. J. Biol. Chem. 276:381-385.

Chapter V

THE UBIQUITIN-PROTEASOME PATHWAY REGULATES GROWTH HORMONE RECEPTOR AVAILABILITY

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ABSTRACT

Growth hormone (GH) promotes not only longitudinal growth in children but is active throughout life in protein, fat and carbohydrate metabolism. The multiple actions of GH start when GH binds to the cell surface-expressed GH receptor. Effectiveness of the hormone depends both on its presence in the circulation and the availability of receptors at the cell surface of target cells. In this study, we examined the role of the ubiquitin-proteasome pathway in regulating GH receptor availability. We show that receptor turnover is rapid, and almost threefold prolonged in the internalisation deficient mutant GHR(F327A). Using a monovalent GH antagonist, B2036, we could quantify the internalisation of the "unoccupied" receptor. By comparing internalisation of the receptor with shedding of the GH-binding protein, we show that in Chinese hamster lung cell lines, internalisation followed by lysosomal degradation is the major pathway for receptor degradation and that the ubiquitin-proteasome pathway controls this process. Inhibition of endocytosis resulted in a 200 percent increase in receptor availability at the cell surface at steady state.

Abbreviations used:

Bq, becquerel; EGF, epidermal growth factor; GH, growth hormone; GHBP, growth hormone binding protein; GHR, growth hormone receptor; JAK, Janus kinase; PDGF, platelet-derived growth factor; STAT, signal transducer and activator of transcription; TACE, tumour necrosis factor-α-converting enzyme; UbE, ubiquitin-dependent endocytosis.

INTRODUCTION

Human growth hormone (GH), a 191 amino acid polypeptide secreted by the anterior pituitary, is the major regulator of postnatal growth and metabolic functions. GH actions on target cells are mediated by the GH receptor (GHR), which is present in cells throughout the body with high expression levels in liver and adipose tissue. The GHR is a single-chain transmembrane glycoprotein of 620 amino acids and member of the cytokine receptor family. Members of this family share limited homology in the extracellular domain, contain a single trans-membrane domain and lack intrinsic kinase activity (Carter Su *et al*., 1996; Waters, 1999). The multiple actions of GH start when one GH molecule interacts with two identical GHR molecules. This binding was hypothesised to occur sequentially, in which the first receptor binds to GH binding site 1, followed by binding of the second receptor to GH binding site 2 (Cunningham *et al*., 1991). The dimerisation of the GHR induces association and activation of the cytosolic tyrosine kinase JAK2, and subsequent tyrosine phosphorylation of JAK2, the GHR, and signal transducers and activators of transcription (STATs) (Argetsinger *et al*., 1993). STAT proteins translocate to the nucleus and transactivate specific genes (Finidori and Kelly, 1995). In addition, GH induces the activation of the mitogen-activated protein kinase (MAP kinase) and the insulin receptor substrate (IRS) pathways (Souza *et al*., 1994; van der Kuur *et al*., 1997).

GH promotes not only postnatal longitudinal growth in children, but functions throughout an individuals's life in protein, fat and carbohydrate metabolism. GH secretion is pulsatile and regulated by two hypothalamic hormones: growth hormone releasing hormone, which stimulates secretion and somatostatin, which inhibits secretion (Muller *et al*., 1999). In the GH-deficient adult, the effects on body composition, fat accumulation, and decreased muscle mass are evident. The effectiveness of a peptide hormone depends equally on its presence in the circulation as well as the availability of receptors at the plasma membrane of target cells. The GHR is synthesised in the endoplasmic reticulum (ER), processed in the Golgi complex, and transported to the plasma membrane. Receptor turnover is rapid with a half-life of about 60 min and hormone-accelerated receptor down-regulation has been observed (Gorin and Goodman, 1985; Ilondo *et al*., 1986; Murphy and Lazarus, 1984). Two mechanisms are known to contribute to receptor turnover: Proteolysis at the cell surface, resulting in shedding of GH-binding protein (GHBP) (Baumann, 1994) and ligand-induced endocytosis. Recently, it was shown that the metalloprotease, tumour necrosis factor-α-converting enzyme (TACE), is involved in the shedding of GHBP (Zhang *et al*., 2000) and that phorbol esters and growth factors can affect GHR proteolysis (Guan *et al*., 2001). In ligand-induced endocytosis, the receptor is also removed from the cell surface, an event that is followed by lysosomal degradation. It was shown that ongoing protein synthesis is important to maintain GH binding capacity (Roupas and Herington, 1988).

The ubiquitin-proteasome pathway is involved in endosomal trafficking of membrane receptors, transporters and channels (reviewed in Hicke, 2001; Strous and Govers, 1999). Several mammalian receptor proteins, such as epidermal growth factor (EGF) receptor (Galcheva-Gargova *et al*., 1995), platelet derived growth factor (PDGF) receptor (Mori *et al*., 1992), *c-kit* receptor (Miyazawa *et al*., 1994), T-cell receptor (Cenciarelli *et al*., 1992), Met receptor (Jeffers *et al.*, 1997) and the GHR (Strous *et al*., 1996) are ubiquitinated in response to ligand binding. Ubiquitination at the plasma membrane usually targets proteins for degradation in the lysosome. The tyrosine kinase adaptor protein, c-Cbl, is an E3 ubiquitin ligase that mediates EGF receptor ubiquitination and promotes sorting of the receptor into multivesicular bodies, thereby attenuating kinase signalling (Levkowitz *et al*., 1998). For the Met receptor, it was recently shown that degradation occurs mainly in lysosomes and that proteasome inhibitors interfere with Met receptor endocytic trafficking (Hammond *et al*., 2001). Ligand-induced endocytosis of the GHR is mediated by the ubiquitin proteasome pathway via a 10 amino acid motif within the cytoplasmic tail (UbE-motif; DSWVEFIELD) (Govers *et al*., 1999) and is inhibited in the presence of proteasome inhibitors (van Kerkhof *et al*., 2000). Most of the above mentioned receptors are long-lived and are rapidly degraded in response to ligand binding and signal-transduction, this process is often referred to as signal down-regulation (reviewed in Waterman and Yarden, 2001; Wiley and Burke, 2001). In contrast, the GHR is short-lived and continuously degraded, whether or not GH is present (Baxter, 1985; Gorin and Goodman, 1985).

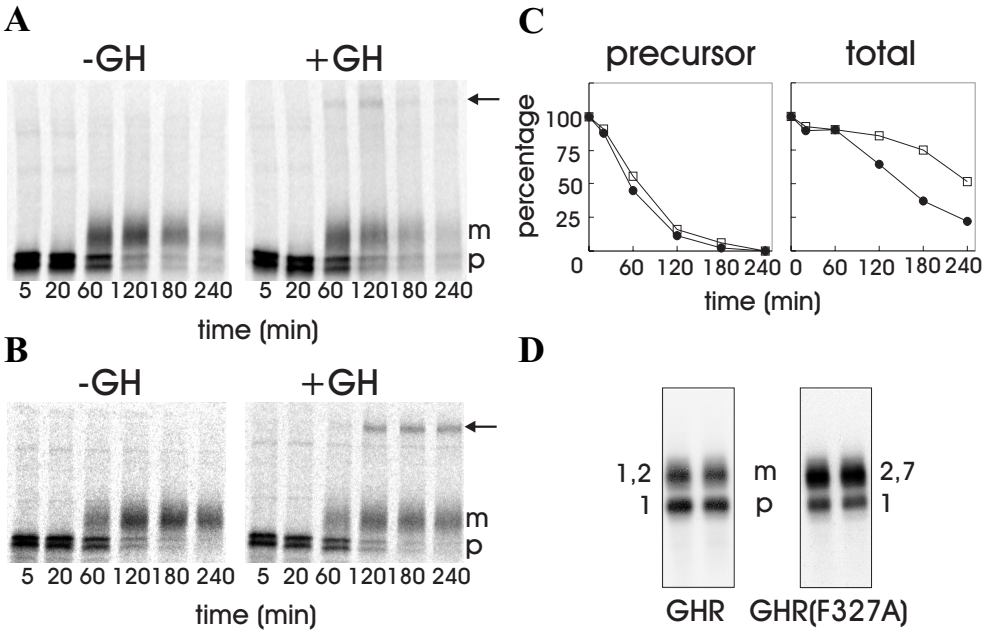
In this study we investigate whether the ubiquitin-proteasome pathway is involved in the rapid turnover of the GHR, thereby controlling the availability of the receptor at the cell surface. By comparing the turnover of the GHR in the absence of ligand with the turnover of an endocytosis-deficient receptor lacking an active UbE-motif, GHR(F327A), we show that the half-life of the mutant GHR is prolonged almost three-fold. A GH antagonist, B2036, which can bind but not properly dimerise the receptors, due to a mutation in binding site 2 (G120K) (Cunningham and Wells, 1991), was internalised by the GHR and delivered to the lysosome for degradation. Internalisation and degradation of B2036 was dependent on an active ubiquitin-proteasome pathway and could be inhibited using proteasome inhibitors. With the use of B2036 as an inactive receptor tag, we measured both GHR internalisation and GHR-proteolysis, generating GHBP. Our data show that ubiquitin proteasome pathway-mediated internalisation is the major determinant in GHR turnover. Thus, the ubiquitin-proteasome pathway controls the time-span of the GHR at the cell surface and its availability for signalling.

RESULTS

Rapid, Ligand-Independent GHR Turnover Requires an Intact UbE-motif

The GHR life cycle is determined by three processes: 1. receptor biosynthesis and transport to the cell surface, 2. GHR internalisation and lysosomal degradation, and 3. GHR proteolysis with shedding of its extracellular domain. Together, these processes determine the surface expression or availability of the receptor for its ligand. The receptor life-cycle was assessed using pulse chase labelling with [^{35}S]methionine followed by immunoprecipitation with anti-GHR antiserum (Fig. 1A). The GHR was synthesised as an 110 kDa glycoprotein precursor (p) and, upon complex glycosylation in the Golgi complex, converted to an 130 kDa mature receptor protein (m) with a half-life of about 55 min (Fig. 1C, left panel). The amount of mature protein was maximal between 60 and 120 min of chase and decreased rapidly thereafter, both in the absence and presence of GH. This result indicates that the receptor turnover rate is high regardless of whether or not it is occupied. At this stage it is important to note that GH accelerated degradation of the receptor, slightly, but reproducibly. The half-life of the receptor, from synthesis to degradation, was estimated at 130 min (Fig. 1C, right panel). When we assume total conversion of the precursor to mature protein and consider the precursor half-life of 55 min, this implies a half-life of about 75 min for the mature protein. To determine the extent to which receptor shedding contributes to its turnover, we used the endocytosis-defective mutant receptor, GHR(F327A) (Allevato *et al*., 1995; Govers *et al*., 1997) (Fig. 1B). The residue Phe-327 is part of the UbE-motif in the GHR cytosolic tail and essential for both ubiquitination and internalisation of the GHR (Govers *et al*., 1999). Like the wild-type receptor, GHR(F327A) was synthesised as an 110 kDa precursor glycoprotein and converted to the 130 kDa mature species. Quantification of the radioactive precursor protein (Fig. 1C, left panel) showed nearly identical kinetics for its disappearance with a estimated half-life of 60 min. Maturation was also comparable, with 40% of the initial synthesised protein converted to the mature species after 60 min of chase. Degradation of the 130 kDa GHR(F327A) was clearly inhibited as compared to the wild-type receptor, resulting in a delayed decrease in total radioactivity (precursor plus mature), with an estimated half-life of 240 min (Fig. 1C, right panel). Thus, when ubiquitin-proteasome pathway dependent endocytosis was impaired, the half-life of the mature receptor was prolonged 2.4 times (180 min). In the presence of GH, a slow migrating form of the receptor could be detected in the top of the gel (see arrow) which is more stable for GHR(F327A). Although the samples were resolved by SDS-PAGE under reducing conditions, this band most likely reflects a GH-dependent disulfide linked GHR dimer, as was reported in IM-9 cells (Zhang *et al*., 1999). The half-life of this complex is similar to that of the 130 kDa band, suggesting that indeed endocytosis mediates its disappearance. The results show that activation of the GHR by GH is not critical for receptor turnover. This observation agrees with results that were obtained

Figure 1. Rapid GHR turnover is dependent on the UbE-motif



A, wild-type GHR and *B*, mutant GHR(F327A) expressing ts20 cells were labelled with [^{35}S]methionine for 10 min and chased in the absence of radioactivity without (-GH) or with 8 nM hGH (+GH) for the indicated times at 30°C. GHR was immunoprecipitated with anti-GHR antibody (anti-T) and separated by SDS-PAGE (7.5%). *p*, precursor GHR (110 kDa); *m*, mature GHR (130 kDa). *C*, radioactivity in precursor protein only (precursor) and in each total lane (total) was quantified and expressed as a percentage of the signal at chase time 5 min. ●, wild-type GHR; □ GHR(F327A) *D*, wild-type GHR and GHR(F327A) expressing ts20 cells were lysed, separated by SDS-PAGE in duplicate and immunoblotted using anti-GHR (mAb5). The individual bands were quantified using a laser equipped densitometer and the amount of mature GHR (m) was expressed compared to the amount of precursor protein (p).

with a GHR mutant that failed to bind JAK2 (C.M. Alves dos Santos, J. Biol. Chem.; *in press*).

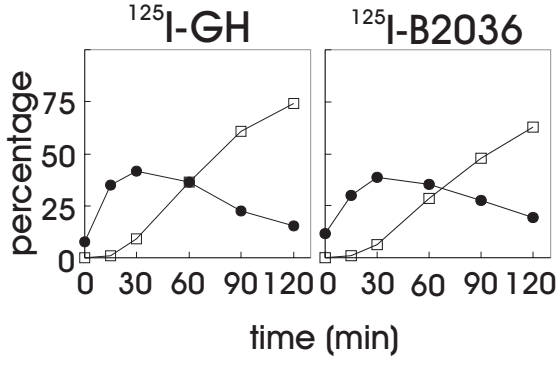
As a second and independent method to determine the half-life of the cell surface receptor, the ratio between precursor and mature protein at steady state was determined. The steady state amount of mature protein reflects the balance between synthesis and degradation. Since the transport and maturation kinetics of the wild-type and endocytosis mutant GHR are similar (see above), differences in ratios are due to differences in the half-lives of the mature GHR. Cells were lysed and aliquots of the lysate were analysed, in duplicate, by western blotting using an antibody against the cytosolic tail (anti-B, not shown) and against the extracellular domain of the receptor (mAb5, Fig. 1D). The individual bands were quantified as explained in materials and methods and the ratio of the mature 130 kDa protein (m) and the precursor (p) was calculated. For

the wild-type receptor, the ratio m/p was 1.2 and for the GHR(F327A) the ratio was 2.7. A direct comparison of the ratios of mutant and wild-type receptor reveals that the half-life of the mature form of the mutant receptor is prolonged 2.2- fold. This comparison agrees with the factor 2.4 (180 versus 75 min), calculated from the pulse chase experiment.

Endocytosis is a Major Factor in the Turnover of the GHR

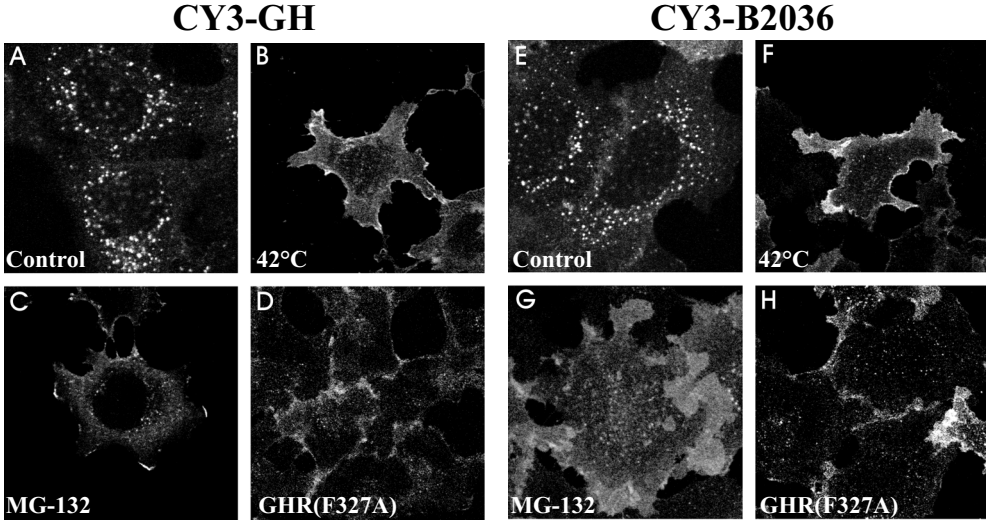
From the previous experiment we concluded that occupancy of the GHR is not an important factor in modulating its turnover rate. Regulation of the turnover in the absence of ligand could be caused by internalisation and subsequent lysosomal degradation of the unoccupied receptor and/or by shedding the extracellular domain of the receptor from the cell surface. To demonstrate internalisation of an "unoccupied" receptor, we used the GH antagonist, B2036, which cannot dimerise the GHR due to a mutation in binding site 2 (G120K). B2036 has an affinity similar to native GH in intact cells (Maamra *et al*., 1999). In our experiments, B2036 did not induce phosphorylation of the GHR, and was able to abolish GH-induced phosphorylation if added in a 5 times molar excess to GH (data not shown). To determine whether the wild-type GHR was able to internalise the antagonist and direct it to the degradative pathway, cells were incubated with either ^{125}I-GH or ^{125}I-B2036 and chased for various times in the absence of ligand. To measure both uptake and lysosomal degradation, internalised radioactivity and TCA-soluble radioactivity were analysed (Fig. 2). Both GH and B2036 were found intracellular after 15 min of chase, with maximum levels of about 40% after 30 min. Thereafter, the amount of intracellular ligand decreased with a concomitant increase of degraded ligand as acid-soluble radioactivity in the medium. Internalisation and degradation of native GH occurred somewhat faster than that of B2036. This result agrees with the observation of a slightly more efficient degradation of the GHR during the pulse chase experiment (Fig. 1) when cells were incubated with GH. A possible expla-

Figure 2. Ligand internalisation and degradation is independent of GHR activation



Wild-type GHR expressing ts20 cells were incubated for 6 min with 8 nM ^{125}I-GH or ^{125}I-B2036. The ligand was removed and the incubation was continued for the indicated times at 30°C. The amount of intracellular and degraded ligand were determined and plotted as a percentage of total radioactivity. The result of one, representative experiment is presented. ●, intracellular; □, degraded.

Figure 3. Endocytosis of B2036 is ubiquitin-proteasome pathway dependent



Wild-type GHR (*A-C* and *E-G*) or GHR(F327A) (*D* and *H*) expressing ts20 cells were incubated with vehicle (*A, E, D* and *H*) or 20 μM MG-132 (*C* and *G*) for 1 h at 30°C or for 1 h at 42°C (*B* and *F*); then Cy3-GH (*A-D*) or Cy3-B2036 (*E-H*) were added for 30 min, and the cells were washed, fixed and the fluorescence was visualised by confocal microscopy. No uptake of B2036 was observed when excess unlabelled GH was added.

nation is that GH activates signal transduction pathways via STAT proteins or MAP kinase which may stimulate receptor mediated endocytosis and thereby stimulate internalisation and degradation to some extent (Ceresa and Schmid, 2000). These results show that B2036 is internalised and degraded through the GHR, confirming that endocytosis and lysosomal degradation contribute to the rapid turnover of the "unoccupied" GHR.

Ligand-Independent Endocytosis Requires an Active Ubiquitin-Proteasome Pathway

Previously, with the use of GHR-expressing ts20 cells bearing a temperature sensitive mutation in the ubiquitin activating enzyme E1, we showed that ligand-induced GHR endocytosis is dependent on an active ubiquitin-proteasome pathway (Strous *et al*., 1996). Here, we addressed the question whether endocytosis of the "unoccupied" receptor, as measured with the antagonist, is also regulated by the ubiquitin-proteasome pathway. The wild-type GHR expressing ts20 cells were incubated with Cy3-GH or Cy3-B2036 at the permissive temperature of 30°C or the non-permissive temperature of 42°C. Incubation at 30°C resulted in abundant label in endosomal and lysosomal compartments (Fig. 3A and 3E), which confirms the internalisation of the B2036, as shown in Fig. 2. Internalisation of both Cy3-GH and Cy3-B2036 was completely inhibited fol-

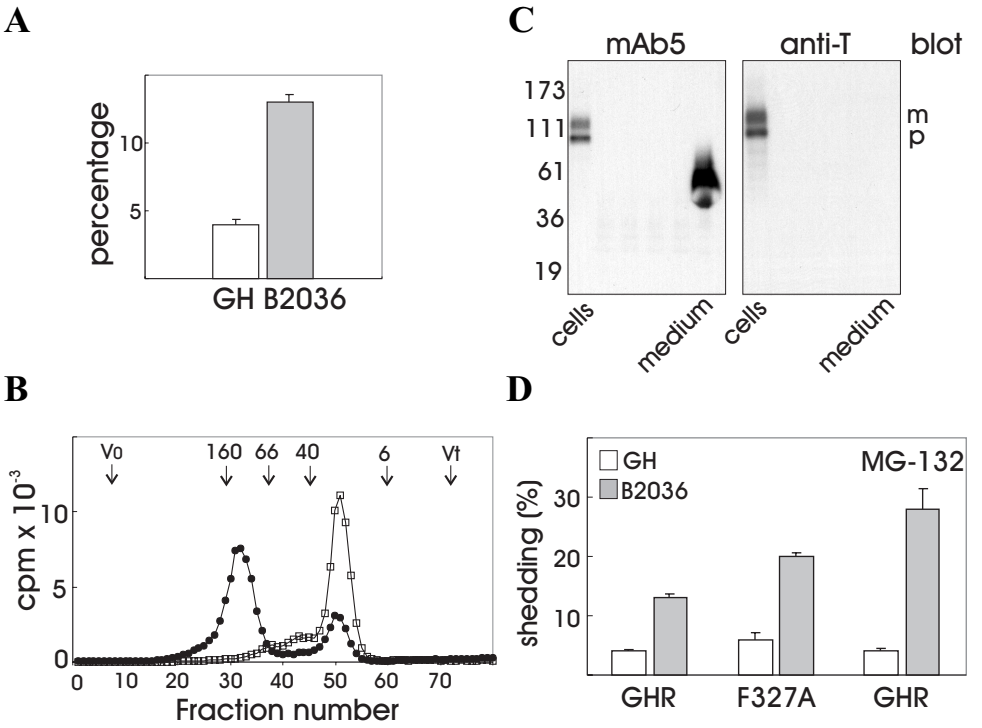
lowing inactivation of the ubiquitin-proteasome pathway at the non-permissive temperature (Fig. 3B and 3F). Therefore, not only does ligand- induced endocytosis but also endocytosis of the "unoccupied" GHR depend on an active ubiquitin-proteasome pathway. Untransfected ts20 cells showed neither Cy3-B2036 binding nor internalisation (not shown).

Ligand-induced endocytosis of the GHR is inhibited in the presence of proteasome inhibitors (van Kerkhof *et al*., 2000). The involvement of the proteasome in endocytosis of the "unoccupied" GHR was demonstrated in the presence of the proteasome inhibitor MG-132. Incubating the cells with MG-132 resulted in complete inhibition of uptake of both GH and the antagonist (Fig. 3C and 3G). The UbE-motif is required for ligand-induced GHR internalisation (Govers *et al*., 1999). Here, we show that mutating the UbE motif, as in GHR(F327A), results in a complete inhibition of uptake of both Cy3-GH and Cy3-B2036, indicating that internalisation of the "unoccupied" receptor requires an intact UbE-motif. In addition, internalisation studies that were performed using ^{125}I-GH and ^{125}I-B2036 demonstrated both uptake and degradation in wild-type GHR expressing cells. Uptake and degradation were inhibited in cells incubated with MG-132 as well as in GHR(F327A) expressing cells (not shown). These results demonstrate that, besides ligand-induced GHR endocytosis, endocytosis of the "unoccupied" GHR depends on the ubiquitin-proteasome pathway.

GHBP Shedding Contributes Little to the Availability of the GHR

Receptor proteolysis by TACE results in shedding of GHBP, and constitutes the third mechanism that may regulate the availability of the GHR at the cell surface. GHBP is the soluble extracellular domain of the GHR and is released, in rabbits, as a 57 kDa protein (Ymer and Herington, 1984). The exact cleavage site has not been established, but is thought to be located at the membrane boundary (Leung *et al*., 1987). Using ^{125}I-B2036, we noticed a considerable amount of TCA-precipitable radioactivity in the medium, which was not observed with GH (Fig. 4A). To further characterise the nature of this radioactivity, the medium was analysed on a gel filtration column and compared to iodinated antagonist which was not incubated with cells. Fig. 4B shows that the majority of the radioactivity, released from the cells, eluted as a complex that was bigger in size than ^{125}I-B2036. To characterise the complex, the medium from B2036-treated cells was immunoprecipitated with anti-GH, and the immunoprecipitate was analysed on western blot. As seen in Fig. 4C, a protein could be detected which reacted with an antibody directed against the extracellular domain of the GHR (mAb5) but not with an antibody against the cytosolic tail (anti-T). The size of the detected protein is the same size as would be expected for the GHBP (57 kDa), indicating that the extracellular domain of the GHR co-immunoprecipitated with B2036. In the cell lysates (cells) both antibodies detected the 110 kDa precursor (p) and the 130 kDa mature (m) forms of the receptor. In the presence of a metalloprotease inhibitor, the release of TCA-

Figure 4. Release of intact B2036 from cells reflects shedding of GHBP



A, wild-type GHR expressing ts20 cells were incubated for 6 min with 8 nM ^{125}I-GH or ^{125}I-B2036. The ligand was removed and the cells were washed and incubated for 30 min at 30°C. The medium was centrifuged and precipitated with 1 volume of 20% TCA for 30 min on ice. The precipitated radioactivity was plotted as a percentage of total radioactivity in cells and medium. The values represent the mean ± S.D. of two experiments. *B,* Cells were incubated with 8 nM ^{125}I-B2036 for 2 h on ice, washed free of unbound radioactivity and incubated for 30 min at 30°C in 1 ml PBS-complete + 0.1% BSA. The medium was collected, centrifuged and the supernatant was analysed on a gel filtration column and compared with ^{125}I-B2036. The radioactivity in each fraction was plotted in cpm. V0 = void volume, fraction 8; Vt = total volume, fraction 72; molecular weight markers 160, 66, 40 and 6 in kDa. ●, medium; □, ^{125}I-B2036, not incubated with cells. Result of one representative experiment is presented. *C*, Cells were incubated with 8 nM B2036 for 2 h on ice, washed free of unbound ligand and incubated for 30 min at 30°C. The medium was collected, centrifuged and the supernatant was immunoprecipitated with the use of anti-GH. The immunoprecipitate (medium) was, together with a cell lysate (cells), separated on SDS-PAGE and immunoblotted with an antibody against the extracellular domain of the GHR (mAb5) and, after reprobing the same blot, against the cytoplasmic tail of the GHR (anti-T). Relative molecular weight standards (M_r x 10^{-3}) are shown at the left. *D*, Wild-type GHR (GHR) or GHR(F327A) expressing cells were incubated with vehicle or 20 μM MG-132 for 1 h, ^{125}I-GH (8 nM) or ^{125}I-B2036 (8 nM) was added and the incubation was continued for 6 min. Cells were washed free of unbound radioactivity and incubated for 90 min at 30°C in the absence of ligand. The medium was analysed as in A and the precipitated radioactivity is plotted as a percentage of total radioactivity in cells and medium. The values represent the mean ± S.D. of two experiments.

precipitated B2036 was inhibited (not shown). Together, these results show that the release of TCA-precipitated B2036 into the medium is due to proteolysis of the GHR, which results in shedding of GHBP.

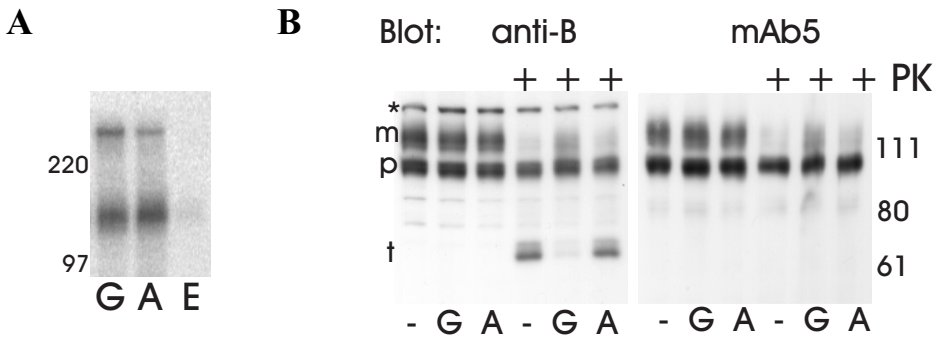
Internalisation and shedding represent two potential mechanisms for regulating receptor availability at the cell surface, and B2036 is the ideal tool to monitor both of these processes. Other methods, like surface biotinylation, often induce artefacts due to changes in binding characteristics of ligands and receptors, and to stress conditions from rapid warming and/or cooling of the cells (Maamra *et al*., 1999). As seen in Fig. 4A, after 30 min of chase, 13% of the ^{125}I-B2036 was bound to GHBP in the medium pellet, while at the same time, 40% was detected intracellular (in Fig. 2). This result indicates that the internalisation pathway is approximately 3-times more efficient in removing GHR from the cell surface than extracellular proteolysis. The cytosolic tail sequence does not play a role in GHBP release, since truncated growth hormone receptor isoforms with intracellular domains of only seven amino acids, lacking both boxes 1 and 2, produce large amounts of GHBP (Iida *et al*., 1999; Ross, 1999). Therefore, it is unlikely that mutations in the UbE-motif of the receptor tail affect GHBP shedding. To define the role of the UbE-motif in both mechanisms, we compared the shedding process in the wild-type GHR and mutant GHR(F327A). Cells were incubated with ^{125}I-GH or ^{125}I-B2036, chased for 90 min in the absence of ligand, and TCA-precipitated radioactivity was measured in the medium (Fig. 4D). The endocytosis-deficient GHR(F327A) mutant showed an increased amount of precipitated ^{125}I-B2036 (22%), compared to 13% in the wild-type GHR expressing cells, while release of intact ^{125}I-GH was low (6 and 4% respectively). This result shows that the prolonged half-life of GHR(F327A) as measured in Fig. 1 is not due to an inhibition of receptor proteolysis. Instead GHBP release increased, a change that was most likely due to an inhibition of endocytosis. For truncated receptor isoforms it is suggested that the lack of internalisation of these receptors can cause increased amounts of GHBP, as more receptor is available for shedding (Ross, 1999). Inhibition of endocytosis with the proteasome inhibitor MG-132 also resulted in an increase in the release of ^{125}I-B2036 (Fig. 4D). As the process of GHR shedding is probably not affected by the presence of B2036, as it is by GH, we conclude that conditions of complete inhibition of GHR endocytosis identify the process of shedding as an important factor for the half-life of the GHR at the cell surface. However, under standard conditions with no GH present, we conclude that internalisation and lysosomal degradation is the main process in GHR turnover. Inhibition of endocytosis, although it increases GHR proteolysis, results in a 200 percent increase in receptors at the cell surface. Thus, for the greater part, the availability of the GHR at the cell surface of Chinese hamster lung cells is regulated by the rate of (ubiquitin proteasome pathway-dependent) endocytosis, and for a minor part by the shedding process.

GH, not B2036, Induces a Conformational Change in the GHR

As argued above, the GH antagonist B2036 is an useful tool to monitor the behaviour of the GHR, as if it is "unoccupied". B2036 binds to the GHR without interfering with either its shedding, or with its endocytosis. Notably, GH-induced dimerisation inhibits GHR proteolysis (Zhang *et al*., 2001). This result raises the issue whether and by what means receptor dimerisation changes the configuration of the GHR, and renders it inaccessible for metalloprotease-mediated proteolysis. To further validate our results with B2036, we examined two important parameters: (i) its behaviour in dimerisation experiments, and (ii) its effect in a protease protection assay. Cells were cross-linked after binding of ^{125}I-GH (G) or ^{125}I-B2036 (A) in wild-type GHR-expressing cells, and the resulting complexes were isolated by immunoprecipitation with either anti-GH (not shown) or anti-GHR (Fig. 5A). Two radio-labelled protein complexes of the same electrophoretic mobility were detected by autoradiography for both ^{125}I-GH and ^{125}I-B2036. These complexes were neither observed in cells which do not express GHR, nor in the presence of excess amounts of unlabelled GH or B2036 (not shown and E in Fig. 5A). The lower molecular weight complexes migrated at a size of 150 kDa, slightly above the mature receptor. Since they were immunoprecipitated with anti-GHR and contained either ^{125}I-GH or ^{125}I-B2036, they most likely represent GHR_1: GH_1 complexes. The complexes at the top of the gel resembled, according to their size, GHR-GH complexes with a stoichiometry of 2:1. Again, both GH- and B2036-containing complexes were of exactly the same size, and therefore, must be of the same stoichiometry. As B2036 is mutated in site 2 and unable to bind the second receptor, these results indicate that the GHR already occurs in a dimeric conformation without a bound ligand. This implies that GH, but not B2036, induces a conformational change in the receptor complex thereby protecting the receptor against the sheddase.

To elucidate this point further, we performed a protease protection assay on the GHR by treating the cells with proteinase K after binding of GH (G) or B2036 (A) (Fig. 5B). When no ligand was present (-), the mature GHR (m) at the cell surface was completely degraded by the protease, leaving only the 110 kDa ER species (p) intact. In addition, digestion of the GHR resulted in a cytosolic tail fragment of 65 kDa (t), which was visible with the anti-tail antibody (anti-B) but not with the antibody against the extracellular domain (mAb5). This result clearly shows that, at steady state, all the mature GHR is present at the cell surface. When cells were incubated on ice with GH, prior to the proteinase K digestion, the mature form was protected against degradation and no formation of the cytosolic tail fragment could be observed. Incubation with B2036 did not protect the GHR against proteinase K digestion. The experiment was repeated with a fivefold higher concentration of ligand and gave the same result. Our data show that GH, not B2036, induces a conformational change in the GHR_2 GH_1 complex, which protects it from digestion by proteinase K. Combined with the observation that GH also

Figure 5. GH, not B2036, protects the GHR from digestion with proteinase K



A, GHR expressing cells were incubated with 8 nM ^{125}I-GH (G), 8 nM ^{125}I-B2036 (A) or 8 nM ^{125}I-B2036 or together with 9 µg/ml unlabelled B2036 (E) for 2 h on ice, washed free of unbound radioactivity with PBS and cross-linked for 30 min on ice with 1 mM DSS in 1 ml PBScomplete. Cells were lysed, centrifuged and the supernatant immunoprecipitated with anti-T. Immunoprecipitates were separated by SDS-PAGE and the radioactivity detected with the use of a phosphorimager. Relative molecular weight standards (M_r x 10^{-3}) are shown at the left. *B*, GHR expressing cells were incubated with 8 nM GH (G), 8 nM B2036 (A) or medium without additions (-) for 2 h on ice, washed with PBS and incubated on ice with (+) or without proteinase K (PK) to digest the extracellular domain of cell surface localised receptors. Cells were lysed, separated by SDS-PAGE and immunoblotted using anti-GHR antibodies directed against the cytosolic tail (anti-B) or the extracellular domain (mAb5). *, aspecific signal also reactive in non-transfected cells; *m*, mature GHR (130 kDa); *p*, precursor GHR (110 kDa); *t*, GHR cytosolic tail (65 kDa). Relative molecular weight standards (M_r x 10^{-3}) are shown to the right.

prevents shedding by TACE (Fig. 4), our results show that the divalent GH, and not the monovalent B2036, shields the GHR/induces proteolytic protection. The results provide strong evidence for a dimerised status of the GHR, be it not protected against TACE. Use of the monovalent GH B2036 is, therefore, a valid tool to monitor the behaviour of the "unoccupied" GHR.

DISCUSSION

In this study we examined the mechanisms which regulate the availability of cell surface GHRs at steady state. In Chinese hamster lung cells, which are stably transfected with GHR, receptor availability is determined by two main factors: 1. endocytosis (75%) and 2. shedding (25%). By using the GH antagonist, B2036, we were able to measure both mechanisms and we could show that constitutive internalisation is mediated by the ubiquitin-proteasome pathway. Inhibition of endocytosis resulted in a 200 percent increase in receptors at the cell surface.

The *availability* of the GHR is determined by its life-time at the plasma membrane. A vast majority of the mature form of the receptor was sensitive to proteinase K diges-

tion, indicating that this form of the GHR resides at the cell surface (Fig. 5B). We used pulse chase labelling with [^{35}S]methionine to calculate the half-lives of the precursor and mature receptor (Fig. 1C). As no ER-degradation of the GHR was detectable (Strous *et al*., 1996), complete conversion of the precursor into mature receptor protein was assumed. For IM-9 cells it was reported that the GHR becomes progressively detergent insoluble in response to GH (Goldsmith *et al*., 1997). By solubilising cell pellets in SDS after cell lysis, no evidence was found that the GHR becomes insoluble in TX-100 in response to GH in Chinese hamster cells, and therefore we assume that the loss of signal is due to receptor degradation. Based on these observations we calculated that the half-life of the mature, cell surface, form of the GHR is 75 min.

Which factors contribute to the rapid turnover of the cell surface receptor? One factor is endocytosis; inhibition of endocytosis results in a 2 - 3-fold prolonged half-life of the unoccupied GHR. Two independent methods were used to compare the half-lives of the mature form of the wild-type GHR with an endocytosis deficient mutant. In the first method we conducted pulse-chase labelling, and in the second method we determined, at steady state, the ratio between the mature and precursor protein. We were permitted to compare the ratio mature/precursor protein since the pulse chase labelling showed that the exit rate from the ER and maturation in the Golgi complex was identical for both receptors. Moreover, the mature form of the mutant receptor is completely proteinase K-sensitive, and thus localised to the cell surface (not shown). Proteolytic shedding of GHBP is the second factor that influences the half-life of the cell surface receptor, and can be blocked by metalloprotease inhibitors (Alele *et al*., 1998). Recent studies demonstrated that GH, but not the GH antagonist G120K, was able to inhibit phorbol ester-stimulated GHR proteolysis (Zhang *et al*., 2001). We show that, by using B2036, it is possible to measure constitutive endocytosis and shedding at the same time. Our experiments revealed that endocytosis contributed 3-times more to turnover of the cell surface receptor than shedding in wild-type GHR expressing cells. If endocytosis and shedding are the only factors determining the turnover of the GHR, than inhibition of shedding in the endocytosis deficient mutant should result in an extended half-life of the mature receptor. Incubation with GH results in inhibition of shedding, but we still observe some degradation of the GHR(F327A) in the presence of GH (Fig. 1B). Incubation of the GHR(F327A) mutant with a metalloprotease inhibitor, increased the half-life considerably (280 min), but could not completely stabilise the mature receptor, indicating that there might be a third factor involved in the degradation (not shown). Precise quantification of this factor is difficult without knowing the type of degradation that may be involved. However, the fact that inhibition of endocytosis [in GHR(F327A)], together with inhibition of shedding (GH or metalloprotease inhibitor), results in a 3 - 4 fold increase in half-life of the cell surface GHR, indicates that these two processes are primarily responsible for degradation. A low amount of degradation

that occurs in the GHR(F327A) mutant expressing cells after inhibition of shedding may be due to ubiquitin-proteasome pathway independent endocytosis, which was previously reported in these cells (van Kerkhof *et al*., 2000).

Since we use transfected cell lines that overexpress the GHR, it was important to establish that the number of GHRs per cell does not affect any of the parameters determining availability. Previously, we showed that increasing concentrations of sodium butyrate (0-10 mM) increase the GHR expression in CMV promotor containing pcDNA3.1 plasmids up to fivefold without affecting the ratio mature/precursor of the GHR (Brown *et al*., 1989; Strous *et al*., 1996). This proves that the half-life of the cell surface receptor remains the same over a considerable range of receptor numbers. The constitutive turnover of the GHR is probably cell type-dependent and is fast in most cell types: in rat adipocytes (T½ = 45') (Gorin and Goodman, 1985), in rat liver (T½ = 40') (Baxter, 1985) and in mouse fibroblasts (T½ = 75') (Murphy and Lazarus, 1984). GH, but not the non-dimerising mutant G120R, induces rapid down-regulation of cell surface receptors in IM-9 lymphocytes (Ilondo *et al*., 1994). In these cells, GH incubation results in irreversible binding and accumulation of long-lived, detergent-insoluble receptors at the cell surface (Goldsmith *et al*., 1997; Zhang *et al*., 1999). Since this down-regulation does not necessarily result in receptor degradation, IM-9 cells may contain a different balance between the factors contributing to GHR down-regulation (Ilondo *et al*., 1992). Our data indicate that, in Chinese hamster ts20 cells, degradation is mainly determined by the ubiquitin-proteasome pathway-dependent internalisation. Future experiments, in which the activity of the ubiquitin pathway can be varied, will provide definitive proof. With the antagonist as a tool to demonstrate receptor internalisation and shedding, it will be possible to determine the contribution of both mechanisms in different cell-types and various conditions.

The current data support a model in which GH induces a conformational change of the GHR, thereby inhibiting the release of GHBP. Earlier studies have suggested that such a conformational change is required for signal transduction (Mellado *et al*., 1997; Rowlinson *et al*., 1998). The fact that GH was able to protect the GHR from digestion by proteinase K, while B2036 did not, supports this model. B2036 was cross-linked to the GHR in a high molecular weight complex which suggests that the GHR can exist as a preformed dimer at the cell surface, as was shown for the erythropoietin receptor (Constantinescu *et al*., 2001; Kubatzky *et al*., 2001; Livnah *et al*., 1999). In this model, the site 2 domain in GH would induce a conformational change in the complex resulting in receptor activation, rather than recruiting the second receptor. The existence of preformed dimers might be beneficial for efficient signalling at low receptor densities. The majority of the complexes we detected after cross-linking represented, as judged from size, however a GH_1GHR_1 complex. We do not know at this point whether this is due to inefficient cross-linking or to a equilibrium at the cell surface between preformed

dimers and real monomeric receptors.

Regulation of receptor number at steady state is of prime importance for GH signal transduction. Here, we provide strong evidence that the ubiquitin-proteasome pathway controls the number of cell surface GH receptors by controlling its constitutive internalisation. Supra-physiological levels of glucocorticoids, whether endogenous (Cushing's syndrome) or exogenous (glucocorticoid therapy), inhibit growth in children. Glucocorticoids activate the ubiquitin-proteasome pathway, and the artificial glucocorticoid, dexamethasone, antagonises cellular GH-action by decreasing GH-binding (Auclair *et al*., 1997; King and Carter Su, 1995), indicating an inversed relation between the activity of the ubiquitin proteasome pathway and GH-binding. In this respect it is interesting to note that the ubiquitin-proteasome pathway is likely the universal system for the degradation of muscle protein induced by starvation (Wing *et al*., 1995), sepsis (Tiao *et al*., 1994), metabolic acidosis (Mitch *et al*., 1994), denervation atrophy (Medina *et al*., 1995), burns (Fang *et al*., 1995) and diabetes mellitus (Price *et al*., 1996). In patients with cancer that undergo a loss of skeletal muscle mass, a condition known as cachexia, the increase in protein breakdown is thought to be associated with an up-regulation of the ubiquitin-proteasome pathway (Tisdale, 2001). The characteristic features of critical illness, like increased protein turnover and a negative nitrogen balance, are partly attributable to resistance to GH (Ross *et al*., 1991). From our results, it is expected that up-regulation of the ubiquitin-proteasome pathway results in the down-regulation of GHR availability, thereby providing a possible explanation for the decreased protein synthesis in these patients.

MATERIALS AND METHODS

Cell Lines and Plasmids

In this study we used the Chinese hamster lung cell line ts20, bearing a thermolabile ubiquitin-activating enzyme E1 (Kulka *et al*., 1988). Ts20 and E36 cells were transfected with a pCB6 construct containing the full length rabbit GHR cDNA sequence (Strous *et al*., 1996). The internalisation-deficient mutant GHR(F327A) was constructed by site-directed mutagenesis, using the unique restriction site *ClaI* and cloned into the CMV-NEO expression plasmid pcDNA3.1 (Invitrogen/Novex) as previously described (Strous *et al*., 1997). For all constructs, stably expressing clonal cell lines were obtained. The ts20 and E36 cells were grown at 30°C in MEMα supplemented with 10% FCS, 4.5 g/l glucose, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.45 mg/ml geneticin. For experiments, cells were grown in 60-mm dishes in the absence of geneticin to a confluence of approximately 75% and 10 mM sodium butyrate was added overnight to increase GHR expression (Strous *et al.*, 1996). Treatment of transfected cells with sodium butyrate did not alter the behaviour of the GHR in any of the parameters examined in this study.

Antibodies and Materials

Polyclonal antibodies to the GHR cytosolic tail were raised in rabbits against fusion proteins of glutathione-S-transferase and GHR peptides consisting of amino acids 271-318 (anti-T) or 327-493 (anti-B) as described before (van Kerkhof *et al*., 2000). Antibody mAb5 recognising the lumenal part of the

GHR was from AGEN Inc. (Parsippany, NJ). Antiserum against human GH was raised in rabbits (Alves Dos Santos *et al*., 2001). MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was from Calbiochem. hGH was a gift from Eli Lilly (Indianapolis, IN). The GH antagonist B2036 is mutated at binding site 2 with G120K. In addition to the G120K mutation, there are 8 additional substitutions that enhance binding site 1 affinity (Cunningham and Wells, 1991). B2036 was kindly supplied by William F. Bennett of Sensus Drug Development Corporation (Austin, TX).

Metabolic Labelling

Cells were grown in 60-mm dishes and incubated in methionine- and cysteine-free MEM, [^{35}S]methionine (3.7 MBq/ml Tran^{35}S Label, 40 Tbq/mmol, ICN, Costa Mesa, CA) was added and the incubation was continued for 10 min at 30°C in a CO_2 incubator. The radioactivity was replaced with medium containing 100 μM unlabelled methionine, 0.1% BSA and chased for 5-240 min. Cells were lysed and samples were immunoprecipitated (see below). Radioactivity was determined with the use of a STORM imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image QuaNT software, version 4.2a.

Ligand Binding, Internalisation and Degradation

^{125}I-human GH and ^{125}I-B2036 were prepared using chloramine T (Strous *et al*., 1996). For internalisation and degradation studies, cells were grown in 12-well plates, washed with MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA and incubated with ^{125}I-GH (8 nM) or ^{125}I-B2036 (8 nM) for 6 min at 30°C. The radioactivity was aspirated and the cells were washed and incubated in medium without ligand. At the indicated times, the medium was collected and precipitated with 1 volume of ice-cold 20% trichloroacetic acid (TCA) for 30 min on ice. Acid-soluble radioactivity was determined in the supernatant after centrifugation and was used as a measurement for degraded ligand. Acid-precipitated radioactivity was determined in the pellet and was used as a measurement for intact ligand. Membrane-associated ligand was removed by acid wash (0.15 M NaCl, 50 mM glycine, 0.1% BSA pH 2.2) for two times 5 min on ice. Internalised ligand was determined by measuring the radioactivity after solubilisation of the acid treated cells in 1 N NaOH with the use of a LKB Compugamma counter. Non-specific radioactivity was determined in the presence of excess unlabelled ligand and subtracted.

Gel Filtration

Cells were grown in 60-mm dishes, washed with MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA, and incubated with ^{125}I-B2036 (8 nM) for 2 h on ice. Cells were washed free of unbound ^{125}I-B2036, 1 ml of PBS-complete (PBS + 1 mM $CaCl_2$ + 0,5 mM $MgCl_2$) + 0.1% BSA was added and the incubation was continued for 30 min at 30°C. The medium was collected, centrifuged, and the supernatant analysed on a HiLoad 16/60 Superdex 200 FPLC column (Amersham Pharmacia Biotech). Fractions (1.2 ml) were collected and counted in the LKB Compugamma counter.

Cross-linking

Cells were grown in 60-mm dishes, washed with MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA and incubated with ^{125}I-GH (8 nM) or ^{125}I-B2036 (8 nM) for 2 h on ice. Cells were washed three times with PBS-complete on ice before 1 mM disuccinimidylsuberate (DSS; Pierce Chemical Co.) freshly dissolved in dimethylsulfoxide (DMSO) was added. After 30 min incubation with the cross-linker, cells were washed with MEMα supplemented with 20 mM Hepes pH 7.4 and 50 mM glycine to quench the unreacted DSS. Cells were lysed and the supernatant was immunoprecipitated.

Proteinase-K Treatment

Cells were grown in 60-mm dishes, washed with MEMα supplemented with 20 mM Hepes pH 7.4 and 0.1% BSA and incubated with GH (8 nM) or B2036 (8 nM) for 2 h on ice. Cells were washed three times with PBS on ice and 700 µl 0.5 mg/ml proteinase K (Roche Molecular Biochemicals) in PBS + 1 mM EDTA was added. Cells were incubated on ice for 30 min and the detached cells were transferred to a test tube. Following centrifugation for 5 min at 300 g at 4°C, the cell pellet was washed 3 times with 1 ml PBS + 2 mM PMSF and lysed in 300 µl lysis buffer. The lysate was centrifuged 5 min 14000 g and 50 µl of the supernatant was boiled with 50 µl of a 2x Laemlli SDS sample buffer containing 40 mM dithiotreitol.

Cell Lysis, Immunoprecipitation, and Western Blotting

Cells were lysed on ice in 0.3 ml of lysis buffer containing 1% Triton X-100, 1 mM EDTA in PBS, with 50 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µM MG-132 and 1 mM PMSF. Immunoprecipitation of the supernatant was carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% BSA in PBS with the various inhibitors. The lysates were incubated with the indicated antibodies for 2 h on ice and immune complexes were isolated using protein A-agarose beads (Repligen Co., Cambridge, MA). The immunoprecipitates were washed twice with the same buffer and twice with tenfold diluted PBS. Immune complexes were subjected to SDS-polyacrylamide gel electrophoresis with the use of the Ready Gel precast gelsystem (Bio-Rad, Hercules, CA) and transferred to polyvinylidenedifluoride (PVDF) paper. The blots were immunostained using the polyclonal GHR antibodies anti-T or anti-B or the monoclonal antibody mAb5 followed by peroxidase conjugated protein A or rabbit-anti-mouse IgG (Pierce Chemical Co.) and detected by enhanced chemiluminescence (Roche Molecular Biochemicals). To reprobe blots, the membranes were incubated for 1 h at room temperature in 0.15 M NaCl, 50 mM glycine pH 2.5 buffer. The efficiency of the stripping procedure was checked and was found to remove >95% of the signal. For quantification, preflashed X-OMAT UV films (Kodak) were used and scanned with a LKB Ultroscan XL Enhanced Laser Densitometer within the linear range of the film and the densitometer and analysed using Gelscan XL, version 2.1 (Amersham Pharmacia Biotech).

Microscopy

Cy3-GH and Cy3-B2036 were prepared using a FluoroLink Cy3 label kit according to the supplier's instructions (Amersham Pharmacia Biotech). Cells grown on coverslips were incubated for 60 min in MEMα supplemented with 20 mM Hepes pH 7.4 + 0.1% BSA and for 30 min with Cy3-GH (1 µg/ml) or Cy3-B2036 (1 µg/ml). Cells were washed with PBS to remove unbound label and fixed for 2 h in 3% paraformaldehyde in PBS. After fixation, the cells were embedded in Mowiol, and confocal laser scanning microscopy was performed using a Leica TCS 4D system.

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REFERENCES

Alele, J., Jiang, J., Goldsmith, *et al*. (1998) *Endocrinology*, **139**, 1927-1935.

Allevato, G., Billestrup, N., Goujon, L., *et al*. (1995) *J. Biol. Chem.*, **270**, 17210-17214.

Alves Dos Santos, C.M., van Kerkhof, P. and Strous, G.J. (2001) *J. Biol. Chem.*, **276**, 10839-10846.

Argetsinger, L.S., Campbell, G.S., Yang, X.N., *et al*. (1993) *Cell*, **74**, 237-244.

Auclair, D., Garrel, D., Zerouala, A. and Ferland, L. (1997) *Am. J. Physiol.*, **272**, C1007-C1016.

Baumann, G. (1994) *J. Endocrinol.*, **141**, 1-6.

Baxter, R.C. (1985) *Endocrinology*, **117**, 650-655.

Brown, D.A., Crise, B. and Rose, J.K. (1989) *Science*, **245**, 1499-1501.

Carter Su, C., Schwartz, J. and Smit, L.S. (1996) *Annu. Rev. Physiol.*, **58**, 187-207.

Cenciarelli, C., Hou, D., Hsu, K.C., *et al.* (1992) *Science*, **257**, 795-797.

Ceresa, B.P. and Schmid, S.L. (2000) *Current Opin. Cell Biol.*, **12**, 204-210.

Constantinescu, S.N., Keren, T., *et al.* (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 4379-4384.

Cunningham, B. and Wells, J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3407-3411.

Cunningham, B.C., Ultsch, M., deVos, *et al*. (1991) *Science*, **254**, 821-825.

Fang, C.H., Tiao, G., James, J., *et al*. (1995) *J. Am. Coll. Surg.*, **180**, 161-170.

Finidori, J. and Kelly, P.A. (1995) *J. Endocrinol.*, **147**, 11-23.

Galcheva-Gargova, Z., Theroux, S.J. and Davis, R.J. (1995) *Oncogene*, **11**, 2649-2655.

Goldsmith, J.F., Lee, S.J., *et al.* (1997) *Amer. J. Physiol-Endocrinol. Met.*, **36**, E932-E941.

Gorin, E. and Goodman, H.M. (1985) *Endocrinology*, **116**, 1796-1805.

Govers, R., ten Broeke, T., van Kerkhof, P., *et al.* (1999) *EMBO J.*, **18**, 28-36.

Govers, R., van Kerkhof, P., Schwartz, A.L. *et al.* (1997) *EMBO J.*, **16**, 4851-4858.

Guan, R., Zhang, Y., Jiang, J., *et al.* (2001) *Endocrinology*, **142**, 1137-1147.

Hammond, D., Urbé, S., Van de Woude, G., and Clague, M. (2001) *Oncogene*, **20**, 2761-2770

Hicke, L. (2001) *Nat. Rev. Mol. Cell Biol.*, **2**, 195-201.

Iida, K., Takahashi, Y., Kaji, H., *et al.* (1999) *J. Clin. Endocrinol. Metab.*, **84**, 1011-1016.

Ilondo, M.M., Courtoy, P.J., *et al.* (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6460-6464.

Ilondo, M.M., Damholt, A.B., Cunningham, *et al*. (1994). *Endocrinology*, **134**, 2397-2403.

Ilondo, M.,Vanderschueren Lodeweyckx,M., *et al.* (1992) *Endocrinology*, **130**, 2037-2044.

Jeffers, M., Taylor, G.A., Weidner, K.M., *et al.* (1997) *Mol. Cell. Biol.*, **17**, 799-808.

King, A.P. and Carter Su, C. (1995) *Endocrinology*, **136**, 4796-4803.

Kubatzky, K.F., Ruan, W., Gurezka, R., *et al*. (2001) *Curr. Biol.*, **11**, 110-115.

Kulka, R.G., Raboy, B., Schuster, *et al.* (1988) *J. Biol. Chem.*, **263**, 15726- 15731.

Leung, D.W., Spencer, S.A., Cachianes, G., *et al.* (1987) *Nature*, **330**, 537-544.

Levkowitz, G., Waterman, H., Zamir, E., *et al.* (1998) *Genes Dev.*, **12**, 3663-3674.

Livnah, O., Stura, E.A., Middleton, S.A., *et al*. (1999) *Science*, **283**, 987-990.

Maamra, M., Finidori, J., Von Laue, S., *et al.* (1999) *J. Biol. Chem.*, **274**, 14791-14798.

Medina, R., Wing, S.S. and Goldberg, A.L. (1995) *Biochem. J*, **307**, 631-637.

Mellado, M., Rodriguez-Frade, J., Kremer, L., *et al.* (1997) *J. Biol. Chem.*, **272**, 9189-9196.

Mitch, W.E., Medina, R., Greiber, S., *et al.* (1994) *J. Clin. Invest.*, **93**, 2127-2133.

Miyazawa, K., Toyama, K., Gotoh, A., *et al.* (1994) *Blood*, **83**, 137-145.
Mori, S., Heldin, C.H. and Claesson-Welsh, L. (1992) *J. Biol. Chem.*, **267**, 6429-6434.
Muller, E.E., Locatelli, V. and Cocchi, D. (1999) *Physiol. Rev.*, **79**, 511-607.
Murphy, L.J. and Lazarus, L. (1984) *Endocrinology*, **115**, 1625-1632.
Price, S.R., Bailey, J., Wang, X., *et al*. (1996) *J. Clin. Invest.*, **98**, 1703-1708.
Ross, R.J., Miell, J., Freeman, E. *et al* (1991) *J. Clin. Endocrinol.*, **35**, 47-54.
Ross, R.J.M. (1999) *Acta Paediatrica*, **88**, 164-166.
Roupas, P. and Herington, A. (1988) *Mol. Cell. Endocrinol.*, **57**, 93-99.
Rowlinson, S., Behncken, S.N., Rowland, J.E., *et al*. (1998) *J. Biol. Chem.*, **273**, 5307-5314.
Souza, S.C., Frick, G.P., Yip, R., *et al*. (1994) *J. Biol. Chem.*, **269**, 30085-30088.
Strous, G.J. and Govers, R. (1999) *J. Cell Sci.*, **112**, 1417-1423.
Strous, G.J., van Kerkhof, P., Govers, R., *et al*. (1996) *EMBO J.*, **15**, 3806-3812.
Strous, G.J., van Kerkhof, P., Govers, R., *et al*. (1997) *J. Biol. Chem.*, **272**, 40-43.
Tiao, G., Fagan, J., Samuels, N., *et al.* (1994) *J. Clin. Invest.*, **94**, 2255-2264.
Tisdale, M.J. (2001) *Front. Bioscience*, **6**, 164-174.
Van Kerkhof, P., Govers, R., *et al*. (2000) *J. Biol. Chem.*, **275**, 1575-1580.
Van der Kuur, J.A., Butch, E.R., Waters, S.B., *et al.* (1997) *Endocrinology*, **138**, 4301-4307.
Waterman, H. and Yarden, Y. (2001) *FEBS Letters*, **490**, 142-152.
Waters, M.J. (1999) *Handbook of Physiology,* ***5,*** Oxford University Press, NY, 397-444.
Wiley, H.S. and Burke, P.M. (2001) *Traffic*, **2**, 12-18.
Wing, S., Haas, A. and Goldberg, A.L. (1995) *Biochem. J.*, **307**, 639-645.
Ymer, S.I. and Herington, A.C. (1984) *Endocrinology*, **114**, 1732-1739.
Zhang, Y., Guan, R., Jiang, J., *et al*. (2001) *J. Biol. Chem.*, **276**, 24565-24573
Zhang, Y., Jiang, J., Black, R.A., *et al*. (2000) *Endocrinology*, **141**, 4342-4348.
Zhang, Y., Jiang, J., Kopchick, J. and Frank, S.J. (1999) *J. Biol. Chem.*, **274**, 33072-33084.

Chapter VI

SUMMARIZING DISCUSSION

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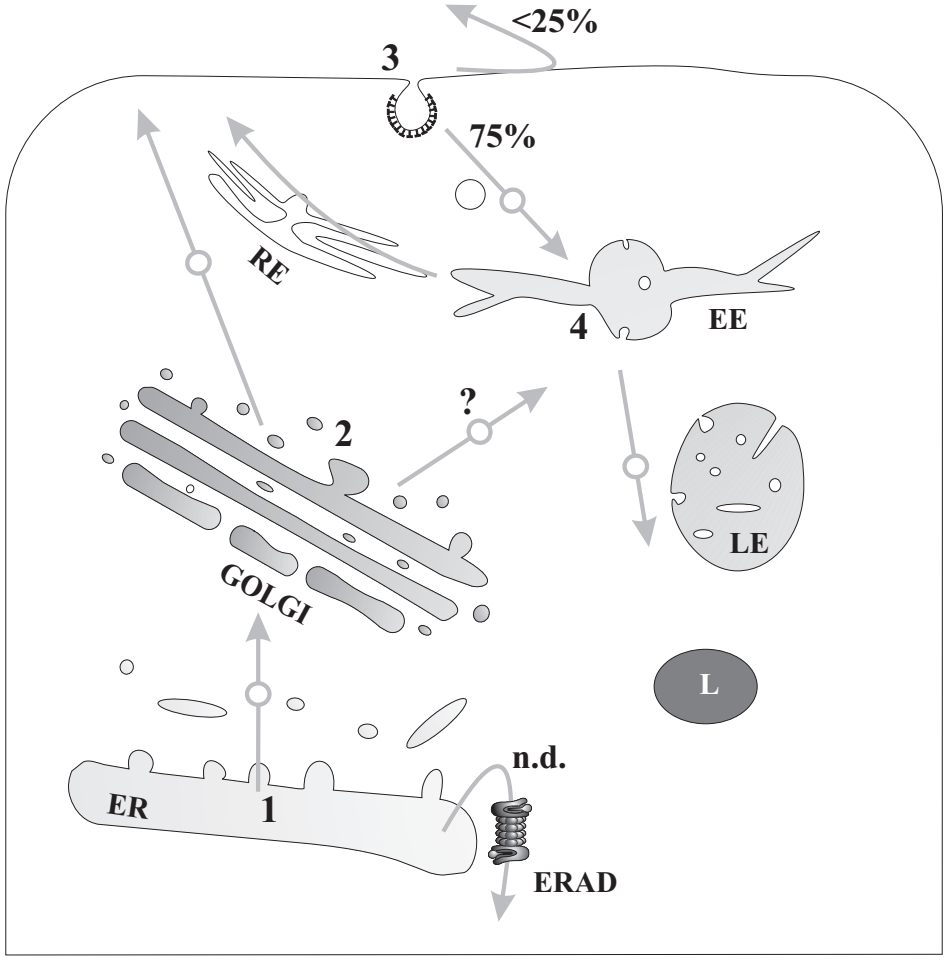
ABSTRACT

The availability of membrane proteins at the cell surface can be regulated at different locations within the cell via different mechanisms (Figure 1). 1) The endoplasmic reticulum (ER) is the site of synthesis of proteins destined to reside at the plasma membrane. The rate of synthesis is largely controlled by the rate at which mRNA is transcribed in the nucleus. The ER-quality control system regulates the exit of properly folded proteins from the ER. Misfolded and incompletely assembled proteins are retained in the ER and eventually degraded. 2) In the *trans*-Golgi network (TGN), proteins can be diverted to the lysosomes without reaching the cell surface. 3) At the plasma membrane, the endocytic machinery can select proteins for endocytosis via clathrin-coated pits or proteins may be subject to proteolysis, resulting in shedding of the extracellular domain. 4) In the sorting endosome, internalized proteins are either recycled back to the plasma membrane or targeted to the lysosome for degradation. Ubiquitination is a post-translational modification that can specifically regulate protein levels via different mechanisms at each of these cellular locations. In transfected Chinese hamster lung cells, GHR availability is determined by two main factors: 1. endocytosis (75%) and 2. shedding (<25%). In this thesis, we show that, at steady state, the level of growth hormone receptors at the cell surface, and thus GHR availability, is regulated by the ubiquitin-proteasome pathway.

Abbreviations used:

CFTR, cystic fibrosis conductance regulator; EGF, epidermal growth factor; ENaC, epithelial sodium channel; EPO, erythropoietin; ER, endoplasmic reticulum; Gal2p, galactose transporter 2p; Gap1p, general amino acid permease1p; GH, growth hormone; GHBP, growth hormone binding protein; GHR, growth hormone receptor; IGF, insulin-like growth factor; IL, interleukin; MPR, mannose-6-phosphate receptor; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SUMO, small ubiquitin-related modifier; TGN, *trans*-Golgi network; TSG, tumor susceptibility gene; UbE, ubiquitin-dependent endocytosis; VEGF, vascular endothelial growth factor.

Figure 1. Schematic representation of intracellular pathways, regulating GHR availability



The major trafficking routes for the GHR in the biosynthetic and the endocytic pathway are indicated by the arrows with spheres, indicating that vesicular transport is involved. ER-associated degradation is not detected for the GHR (n.d.). GHR availability is determined by endocytosis (75 %) and by shedding of the extracellular domain (< 25%). For explanation of the individual steps, see text.
ER, endoplasmic reticulum; ERAD, ER-associated degradation; RE, recycling endosome; EE, early endosome; LE, late endosome; L, lysosome.

Transport of Newly Synthesized GHR to the Cell Surface

ER-associated degradation is part of a quality control mechanism that can regulate the amount of proteins at the cell surface. Studies on the erythropoietin (EPO)-receptor showed that only a small amount of newly synthesized receptor is expressed at the cell surface, even when cells are engineered to overexpress the receptor [1]. Proteasome inhibitors prevented cell surface receptor down-regulation upon stimulation with EPO,

possibly by inhibiting the degradation of newly synthesized receptors and enabling their transport to the cell surface [2]. Could this mechanism be applicable to regulation of GHR availability? Our data provide evidence that ubiquitin-dependent ER-associated degradation does not regulate the amount of GHR transported to the cell surface because i) the GHR was not degraded in the presence of brefeldine A, an inhibitor of protein export out of the ER [3], ii) almost all of the newly synthesized precursor form of the receptor was converted into the mature protein [see chapter III of this thesis], iii) transport of newly synthesized receptors to the cell surface was similar for wild-type GHR and mutant receptors that cannot be ubiquitinated [chapter IV, GHR(399)(K271-362R) and chapter V, GHR(F327A)], and iv) proteasome inhibitors did not increase the amount of 110 kDa GHR precursor protein [chapter III].

Degradation in the ER is not the only mechanism that regulates protein transport to the cell surface in the biosynthetic pathway. A mechanism for diverting newly synthesized proteins from the flow of proteins to the cell surface, involves sorting at the TGN. A well studied example is the delivery of lysosomal enzymes via mannose-6-phosphate receptors (MPRs) in clathrin-coated vesicles to endosomes, thereby avoiding the cell surface [reviewed in: 4]. Recent studies suggest a role for ubiquitination in vacuolar targeting in yeast. Intracellular trafficking of the tryptophan permease Tat2p from the Golgi complex to the vacuole can be blocked by mutation of lysine residues that may act as ubiquitin acceptor sites [5]. The general amino acid permease (Gap1p) is transported from the TGN to the plasma membrane or to the vacuole, depending on the nitrogen source. A polyubiquitin signal on Gap1p specifies its intracellular targeting to the vacuole [6]. Regarding the GHR we have no indications that sorting in the TGN regulates its cell surface expression in unstimulated conditions. We do observe a slightly more efficient degradation of the mature GHR after incubation with GH [chapter V]. The GH antagonist B2036, does not induce this accelerated degradation, indicating that signal transduction may be involved (unpublished results). Our results show that endocytosis is the main mechanism leading to receptor degradation. Endocytosis is, most likely, accelerated after incubation with GH resulting in a more efficient degradation. However, we can not exclude that (part of) the GH-induced accelerated degradation is due to sorting of the GHR in the TGN to the lysosomes.

Transport of the GHR in the Endocytic Pathway

After reaching the cell surface, many plasma membrane proteins are endocytosed and ultimately degraded in the lysosome. The mechanism that regulates ubiquitin-dependent GHR endocytosis appears to be different from what has been described for other plasma membrane proteins so far. In *Saccharomyces cerevisiae*, internalization of plasma membrane proteins requires the ubiquitination of their cytosolic tail and it is thought that the ubiquitin-moiety itself carries the internalization information.

Ubiquitination of the GHR itself is not required, suggesting that ubiquitination of accessory proteins might be involved [Chapter III, IV and 7].

The epithelial sodium channel (ENaC) is, other than the GHR, the only mammalian plasma membrane protein of which it was shown that the number of channels at the cell surface is regulated by the ubiquitin-proteasome pathway. ENaC is recognized by the ubiquitin-ligase Nedd4 via a PY-motif and it was shown that ubiquitination of a cluster of lysine residues at the N-terminus of the γ-chain is important for its function. The PY-motif is not present in the GHR cytosolic domain, the GHR contains the UbE-motif that is essential for ubiquitination and internalization. By database analysis, potential sequences resembling the UbE-motif were found in the prolactin receptor, the platelet-derived growth factor (PDGF)-receptor, the vascular endothelial growth factor (VEGF) receptor and the insulin-responsive glucose transporter Glut4 [7]. Glut4 is modified by the ubiquitin-like protein, SUMO-1 (Small ubiquitin-related modifier). The SUMO-conjugating enzyme mUbc9 binds directly to a C-terminal, 11 amino-acid, sequence of Glut4, not resembling the UbE-like motif. Overexpression of mUbc9 resulted in an 8-fold increased Glut4 abundance, leading to enhanced transport stimulation by insulin [8]. Ubiquitination of the prolactin receptor and the PDGF-receptor is detected, but no evidence is available that the UbE-motif is involved in these proteins. Interestingly, residue Tyr579 in the potential UbE-motif of the PDGF-receptor, corresponding to Phe327 in the GHR, was demonstrated to be involved in PDGF internalization [9].

The receptor tyrosine kinases (RTKs) represent another group of proteins whose cell surface expression may be regulated by ubiquitination. Ubiquitination may occur at the cell surface or in the sorting endosome and in most cases the adaptor protein c-Cbl has been identified as the ubiquitin-ligase. In this group of proteins, phosphorylation is essential for recruitment of c-Cbl via its SH2-domain. In contrast, ligand-induced tyrosine-phosphorylation of the GHR is not required for ubiquitin system-dependent internalization [chapter V and 10].

With the use of a cell line, carrying a temperature-sensitive ubiquitin-activating enzyme (E1), it was demonstrated that GHR internalization depends on an intact E1, both in the presence and absence of GH [chapter V and 3]. The same cell line was used to show that internalization of the β-chain of the interleukin (IL)-2 receptor [11] or TrkA, the receptor tyrosine kinase for nerve growth factor [chapter V] does not require an active ubiquitin-conjugation system. Internalization of a C-terminal truncated GHR at amino acid 349, is mediated by a di-leucine motif and does also not require an active ubiquitin system for endocytosis [12]. Is it possible that the full-length receptor is truncated at the plasma membrane in a ubiquitin-dependent mechanism and subsequently internalized via the di-leucine motif? This possibility is unlikely since i) mutation of the di-leucine motif in the full-length receptor does not abolish its internalization [12], and ii) internalization of the truncated GHR occurs with faster kinetics than the ubiquitin-sys-

tem mediated internalization of the full-length receptor [compare chapter IV and V]. These two observations suggest that different mechanisms mediate di-leucine- and ubiquitin system-dependent endocytosis.

Internalized molecules are rapidly delivered to early endosomes, also known as sorting endosomes. With the use of a truncated receptor, whose internalization is independent of the ubiquitin-proteasome pathway, we could show that the ubiquitin proteasome pathway is involved in the sorting of internalized proteins to the lysosomes [chapter IV]. In the GHR, the UbE-motif is required in this sorting step, and with the use of proteasome inhibitors, we could show that the ubiquitin-proteasome pathway is involved in degradation of both GH and GHR. Comparable inhibition of degradation of internalized receptors in the presence of proteasome inhibitors is reported for the IL-2 receptor [11, 13], the EGF-receptor [14] and trkA [chapter IV]. Inhibition of the proteasome results in recycling of membrane proteins, suggesting that the ubiquitin-proteasome pathway regulates the sorting machinery. Several potential factors of the ubiquitin-proteasome pathway have been implicated in endosomal sorting to lysosomes. In *S. cerevisiae*, the F-box protein Rcy1p is involved in endocytic membrane traffic and recycling [15] and the de-ubiquitinating enzyme Doa4p acts at the prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole [16]. The mammalian tumor susceptibility gene *tsg101* , which encodes the homologue of vps23p, is required for the delivery of cargo proteins to late endosomal compartments. Both vps23 and tsg101 have a similar predicted domain structure, with an N-terminal domain that is homologous to ubiquitin-conjugating enzymes (E2s), but which lacks the active site cysteine. This feature is reminiscent of the ubiquitin-conjugation enzyme variant (UEV)-family of proteins which are thought to regulate E2-activity [17]. These are all potential components of the sorting machinery, possibly involved in selecting cargo for the degradative pathway. Whether and how they connect to their cargo, which protein-protein interactions are essential, what is the role of ubiquitination and the proteasome, and which other factors may be required, are important questions that await answers.

The Role of GHR Ubiquitination

Mutation of the lysine residues in the truncated GHR(399) results in a receptor that is not ubiquitinated [chapter III]. Unlike mutation of the UbE-motif, mutation of the lysine residues does not increase the half-life of the receptor [chapter IV and V]. In contrast, mutation of the cytosolic lysine residues in the IL-2 R β-chain, does not affect internalization but does increase the cell surface half-life of this mutant, suggesting a role for ubiquitination in sorting to lysosomes [11]. Endocytosis of the lysine-less, truncated GHR is ubiquitin-proteasome pathway dependent, demonstrating that ubiquitination of the GHR itself is not required [chapter III and 7]. What is the purpose of GHR-

ubiquitination ? At this moment the answer to this question is not clear since the lysine-less, non-ubiquitinated, receptor behaves the same as the wild-type receptor. This observation suggests that ubiquitination of the receptor is a by-stander effect. The ubiquitination machinery is probably active, via the UbE-motif or ancillary proteins, close to the rim of the clathrin-coated pit [chapter II] and may be involved in ubiquitination and degradation of factors that connect the GHR to the endocytic machinery.

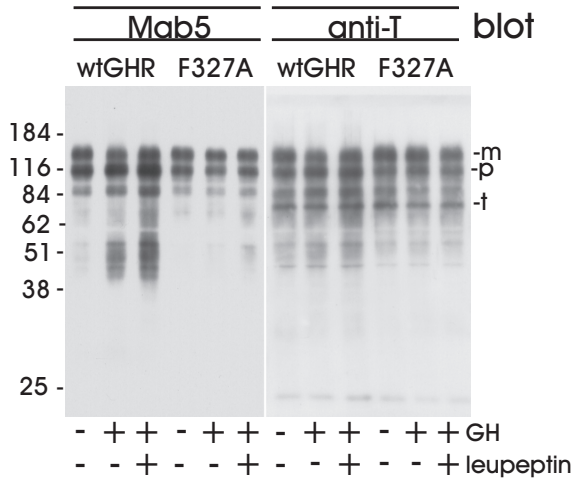
Why does GHR ubiquitination increase after GH-binding? Incubation with GH results in a conformational change of the receptor [chapter V]. This conformational change in itself is not required for recognition by components of the ubiquitin-system since the GHR is ubiquitinated in the absence of GH. GH prevents the GHR from shedding [chapter V] which results in more receptors available for endocytosis, and therefore, could result in increased ubiquitination.

The Role of the Proteasome

A) <u>At the cell surface</u>

Genetic experiments in yeast, with proteasome defective mutants, indicated that the proteasome is not involved in the degradation of ubiquitinated plasma membrane proteins. In mammalian cells, proteasome inhibitors were used to examine the role of the proteasome in the degradation of polyubiquitinated plasma membrane proteins. The degradation of many ubiquitinated membrane proteins is inhibited in the presence of specific proteasome inhibitors [chapter I]. It is suggested that the use of proteasome inhibitors bears the risk of exhausting cells of free ubiquitin, which may result in reduced ubiquitination of target proteins, and thereby throw doubt on a direct role of the proteasome in their degradation [18, 19]. Indeed, incubation of GHR transfected cells with proteasome inhibitors results in the accumulation of non-ubiquitinated GHRs (unpublished results, van Kerkhof). However, for the Met-receptor and the IL-2 receptor β-chain, incubation with proteasome inhibitors results in accumulation of ubiquitinated forms of these proteins, suggesting a direct role for proteasomes, together with lysosomes, in the turnover of these proteins [11, 20]. Since internalization of the truncated GHR(369) in the presence of proteasome inhibitors [chapter III] is dependent on an active ubiquitin-activating enzyme E1 [12], it is unlikely that free ubiquitin is exhausted in these cells. Thus, we examined the possibility that the cytosolic tail of the GHR is degraded by the proteasome, while the lumenal part is degraded within the lysosome. To investigate the formation of intermediate degradation products of the GHR after ligand-induced endocytosis, cells were incubated for 6 h with GH in the absence and presence of the lysosomal protease inhibitor, leupeptin (Figure 2). Cell lysates were analyzed with antibodies against the extracellular domain of the GHR (Mab5) and against a membrane proximal domain of the cytosolic tail (anti-T). In the presence of GH, low molecular weight species of the receptor were detected with Mab5, indicating partial

Figure 2. Degradation products of the GHR



Wild-type GHR or GHR(F327A) expressing cells were incubated for 6 h with GH in the presence of 100 μM leupeptin as indicated. Western blots of cell lysates were detected with antibodies against the extracellular (Mab5) or cytosolic (anti-T) domain of the GHR. m, mature GHR (130kDa); p, precursor GHR (110 kDa); t, cytosolic tail (70 kDa). Relative molecular weight standards (M_r x 10^{-3}) are shown at the left.

degradation of the wild-type GHR. When the internalization mutant GHR(F327A) was used, no such intermediates were present. Leupeptin increased the amount of intermediate degradation products detectable with the Mab5 antibody, indicating the involvement of lysosomes in the degradation of the lumenal part of the receptor. The majority of the degradation products reactive with Mab5, migrated on the SDS gel as proteins of 50-60 kDa, similar in size to the circulating GH-binding protein (50-60 kDa). The degradation products, accumulated in the presence of GH, and detected with Mab5, did not show up with antibodies directed against the cytosolic tail (anti-T). Given their sizes and their reactivity towards the different antibodies, our data indicate that most of the cytosolic tail is removed from the 50-60 kDa degradation products. One extra band (designated "t") as compared to the Mab5 detection could be observed in the anti-T western blot. Most likely, this band represents the remaining cytosolic tail of the receptor after the proteolytic release of GH-binding protein [chapter V]. These data demostrate that the lysosome is involved in the degradation of the extracellular domain of the GHR, and suggest that the proteasome is involved in degradation of the cytosolic tail. Detection of proteasomal degradation intermediates may be very difficult due to degradation intermediates of various lengths, which are not easily detectable by immunoblotting. Indeed, both with Mab5 and anti-T, a smear of immunoreactive proteins was detected upon GH incubation, primarily in the lanes containing the wild-type receptor. In this smear no epitopes were detectable with the antiserum against the C-terminal part of the tail (not shown). This result indicates a gradual degradation of the receptor cytosolic tail during or after its endocytosis. To define the role of the proteasome in GHR endocytosis, two possibilities have to be distinguished: 1) receptor degradation begins at the cell surface, before endocytosis, or 2) receptor degradation begins after

endocytosis. In the first scenario, the proteasome would be required to truncate the GHR cytosolic tail, thereby enabling internalization. This would explain why a truncated receptor is internalized in the presence of proteasome inhibitors [chapter III]. Recently, we could detect a small amount of full-length receptor intracellular and bound to GH, indicating that degradation of the cytosolic tail begins shortly after its endocytosis [21]. In that case, another possible role for the proteasome might be envisioned, in which the GHR cytosolic tail, via the amino acid sequence 369-399, binds an additional factor that has to be removed by the proteasome before endocytosis can proceed.

B) At the sorting endosome

Inhibition of endocytosis by proteasome inhibitors has been demonstrated in both the GHR (this thesis) and in the Met-receptor [22]. In many more reported cases, proteasome inhibitors interfere with the degradation of internalized receptors (see above and chapter I). In case of the GHR, a truncated receptor is internalized but not degraded in the presence of proteasome inhibitors [chapter III]. If the UbE-motif is mutated, or if proteasomal activity is inhibited, internalized receptors are recycled to the plasma membrane as opposed to being sorted to the lysosome [chapter IV]. Rocca and colleagues showed that a chimeric interleukin receptor, $\alpha_Y\beta_{18\text{-}27}$, which is targeted for lysosomal degradation, co-localized with the transferrin receptor after incubation with lactacystin [11]. These data remain consistent with a role for the proteasome in regulating the activity of the sorting machinery. Different receptors may recruit the ubiquitin conjugation machinery via various motifs, like the UbE-motif in the GHR, the phosphorylation of Y1045 in the EGF-receptor [23], a 10 amino-acid motif in the IL2-receptor β chain [24] and unidentified motifs in the Met receptor and TrkA. Mutation of these motifs, or inhibition of the proteasome, could result in inhibition of sorting at the limiting membrane of the multi vesicular body and, as a consequence, an inhibition of degradation of the receptor-ligand complex.

Regulating GHR Availability

Plasma membrane proteins are essential for cells to communicate with the outside world, as well as to sense signals and changing conditions. The activity of plasma membrane proteins can be regulated by their number at the cell surface. Upon various extracellular stimuli or changing environmental conditions, these proteins can be quickly removed from the cell surface by endocytosis. In yeast, the presence of permeases at the cell surface, such as Gap1p or the galactose transporter (Gal2p), is regulated by modulation of their internalization rate, which depends on nutrient availability. In mammalian cells, ENaC is under tight hormonal control by aldosterone and vasopressin, and ENaC activity is regulated by either altering the channel open probability or the number of channels at the cell surface. Cellular uptake of glucose is regulated via the insulin receptor that controls the cell surface availability of the glucose transporter, Glut 4. The

ubiquitin-proteasome pathway is involved in the down-regulation of many plasma membrane proteins. In this thesis we established a role for the ubiquitin-proteasome pathway in regulating the number of GHRs at the cell surface. Does this observation imply that cell surface levels of GHR vary upon altered physiological conditions? Nutritional state profoundly affects all levels of the GH/insulin-like growth factor-1 (IGF-1) axis, e.g. fasting is always associated with decreased IGF-1 levels. Changes due to fasting, represent a condition known as GH resistance. High GH and low IGF-1 levels are characteristic for GH resistance, indicating reduced signaling via the GHR [25]. Chronic liver disease, one of the classical forms of acquired GH resistance, is accompanied by low levels of GHR [26, 27]. Also in post-surgical patients, an acute fall of IGF-1 at the time of surgery is observed and clinical conditions as anorexia and diabetes demonstrate high GH and low IGF-1 levels. In many clinical studies, the serum measurement of GH-binding protein (GHBP) is used as a index of GHR expression and GH responsiveness [28]. Accordingly, it was shown that in malnutrition, anorexia, diabetes and liver cirrhosis, the GHBP concentrations are markedly decreased, again indicating decreased expression of the GHR. High GHBP (hence, increased expression of GHR) and high IGF-1 levels are observed in obesity. These examples indicate that GHR expression levels indeed change upon altered physiological conditions. Malnutrition and other catabolic states that have been associated with acquired GH insensitivity, are accompanied by reduced GHBP concentrations, increased protein turnover, and a negative nitrogen balance [29, 30]. Protein degradation exceeds synthesis in catabolic diseases and muscle atrophy will occur as seen after nerve injury and in starvation, cancer cachexia, sepsis, renal failure, burns and AIDS. Several studies have suggested that the enhanced proteolysis in these various pathological states is primarily due to activation of the ubiquitin-proteasome pathway and proteasome inhibitors have been shown to reduce muscle wasting [31]. From our results, it is expected that the up-regulation of the ubiquitin-proteasome pathway results in a down-regulation of the cell surface expression of the GHR, and hence, in decreased GHR availability. Future research will be directed towards identification of the molecular mechanisms that connect the ubiquitin-proteasome pathway with GHR down-regulation. The high selectivity and specificity of the system enable the development of drugs that increase GHR availability in critically ill patients and reverse catabolic into anabolic conditions.

REFERENCES

1 Watowich, S. S. (1999) Int. J. Biochem. Cell Biol. **31**, 1075-1088.

2 Verdier, F., Walrafen, P., Hubert, N., Chretien, S., Gisselbrecht, S., Lacombe, C. and Mayeux, P. (2000) J. Biol. Chem. **275**, 18375-18381.

3 Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A. L. (1996) EMBO J. **15**, 3806-3812.

4 Lemmon, S. K. and Traub, L. M. (2000) Curr. Opin. Cell Biol. **12**, 457-466.

5 Beck, T., Schmidt, A. and Hall, M. N. (1999) J. Cell Biol. **146**, 1227-1237.

6 Helliwell, S., Losko, S. and Kaiser, C. (2001) J. Cell Biol. **153**, 649-662.

7 Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L. and Strous, G. J. (1999) EMBO J. **18**, 28-36.

8 Giorgino, F., de Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K. C.and Smith, R. J. (2000) Proc. Natl. Acad. Sci. USA **97**, 1125-1130.

9 Mori, S., Ronnstrand, L., Claesson-Welsh, L. and Heldin, C. H. (1994) J. Biol. Chem. **269**, 4917-4921.

10 Alves Dos Santos, C. M., ten Broeke, T. and Strous, G. J. (2001) J. Biol. Chem. **276**, *in press.*

11 Rocca, A., Lamaze, C., Subtil, A. and Dautry-Varsat, A. (2001) Mol. Biol. Cell **12**, 1293-1301.

12 Govers, R., van Kerkhof, P., Schwartz, A. L. and Strous, G. J. (1998) J. Biol. Chem. **273**, 16426-16433.

13 Yu, A. and Malek, T. (2001) J. Biol. Chem. **276**, 381-385.

14 Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B. and Yarden, Y. (1998) Genes Dev. **12**, 3663-3674.

15 Wiederkehr, A., Avaro, S., Prescianottobaschong, C., Haguenauer-Tsapis, R. and Riezman, H. (2000) J. Cell Biol. **149**, 397-410.

16 Amerik, A. Y., Nowak, J., Swaminathan, S. and Hochstrasser, M. (2000) Mol. Biol. Cell **11**, 3365-3380.

17 Babst, M., Odorizzi, G., Estepa, E. J. and Emr, S. D. (2000) Traffic **1**, 248-258.

18 Swaminathan, S., Amerik, A. Y. and Hochstrasser, M. (1999) Mol. Biol. Cell **10**, 2583-2594.

19 Schubert, U., Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F., Bennink, J. R.,Krausslich, H. G. and Yewdell, J. W. (2000) Proc. Natl. Acad. Sci. USA **97**, 13057-13062.

20 Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S. and Vandewoude, G. F. (1997) Mol. Cell. Biol. **17**, 799-808.

21 Alves Dos Santos, C. M., van Kerkhof, P. and Strous, G. J. (2001) J. Biol. Chem. **276**, 10839-10846.

22 Hammond, D., Urbé, S., Van de Woude, G. and Clague, M. (2001) Oncogene **20**, 2761-2770.

23 Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K.,Reiss, Y., Ciechanover, A., Lipkowitz, S. and Yarden, Y. (1999) Mol. Cell **4**, 1029-1040.

24 Subtil, A., Rocca, A. and Dautry-Varsat, A. (1998) J. Biol. Chem. **273**, 29424-29429.

25 Ross, R. J. M. (2000) Int. J. Obesity **24**, S92-S95.

26 Chang, T., J., L. and Yu, S. (1990) Hepatology **11**, 123-126.

27 Shen, X., Holt, R. and Miell, J. (1998) J. Clin. Endocrinol. Metab. **83**, 2532-2538.

28 Barnard, R. and Waters, M. J. (1997) J. Endocrinol. **153**, 1-14.

29 Rosenbloom, A. L. (2000) Endocrine **12**, 107-119.

30 Ross, R. J., Miell, J. and Freeman, E. (1991) J. Clin. Endocrinol. **35**, 47-54.

31 Tawa, N. E., Odessey, R. and Goldberg, A. L. (1997) J. Clin. Invest. **100**, 197-203.

Nederlandse samenvatting

Groeihormoon wordt gemaakt in de hypofyse-voorkwab en via de bloedbaan door het lichaam getransporteerd. Het meeste groeihormoon wordt aangemaakt bij pasgeborenen, na het twaalfde levensjaar gaat de groeihormoonproductie omlaag. Een verminderde groeihormoonproductie wordt gezien als een belangrijke factor bij veroudering en achteruitgang van veel lichamelijke functies op hogere leeftijd. Groeihormoon is namelijk niet alleen belangrijk voor de lengtegroei tot aan de puberteit, maar bevordert gedurende het gehele leven de vitaliteit. Groeihormoon, als een van de meest essentiële "onderhoudshormonen", is van belang voor het stimuleren van het herstel van beschadigd weefsel, voor de opbouw en instandhouding van de spiermassa en voor de verbranding van overtollig vet. Een tekort aan groeihormoon (groeihormoondeficiëntie) resulteert in groeivertraging (dwerggroei) en onder andere in een overmaat aan vetweefsel, met name rond de buik. Overproductie van groeihormoon, een klinisch syndroom dat acromegalie wordt genoemd, leidt onder andere tot vergrote handen en voeten en een verdikte huid. Voor de signaalverwerking van groeihormoon is een mechanisme nodig dat de aanwezigheid van het hormoon in het bloed opmerkt. Op de celwand (plasmamembraan) van alle cellen bevinden zich receptoren, eiwitten die uitsluitend één bepaald hormoon herkennen. De groeihormoonreceptor op het celoppervlak van bijvoorbeeld lever-, vet-, spier- of botcellen, bindt het in het bloed circulerende groeihormoon en geeft vervolgens een signaal door naar de celkern. Het gevolg is dat de in het DNA opgeslagen informatie wordt vertaald in de aanmaak van díe eiwitten, die de door het hormoon opgedragen taak uitvoeren. Het door de hypofyse gegenereerde groeihormoon kan dus alleen een signaal doorgeven als er voldoende groeihormoonreceptoren op de celwand van de ontvangende cellen beschikbaar zijn. In het in dit proefschrift beschreven onderzoek hebben wij ontdekt, hoe het aantal groeihormoonreceptoren aan het celoppervlak gereguleerd wordt.

Om de hoeveelheid van een bepaald eiwit te reguleren kan een cel de aanmaak (synthese) van dit eiwit beïnvloeden maar ook de afbraak (degradatie). De balans tussen synthese en degradatie bepaalt uiteindelijk de absolute hoeveelheid van ieder individueel eiwit. Een verandering van omstandigheden, zoals bijvoorbeeld wijziging van voedingspatroon, veroudering, of ziekte zal deze balans beïnvloeden en dus de eiwitsamenstelling en het functioneren van de cel. Een belangrijk eiwitafbraak mechanisme in de cel is het ubiquitine-proteasome systeem. In het jaar 2000 werd in Amerika de Lasker prijs voor fundamenteel medisch onderzoek uitgereikt aan Avram Hershko, Aaron Ciechanover en Alex Varshavsky voor de ontdekking van dit systeem. Zij toonden aan dat eiwitten door dit systeem op een gecontroleerde manier worden afgebroken. Het ubiquitinesysteem is van groot belang bij onder meer de celdeling, ontstekingsreacties, immuniteit en het vermogen om schadelijke eiwitten uit het lichaam te ver-

wijderen. Eiwitten die moeten worden afgebroken, worden eerst gemerkt met een klein eiwit dat ubiquitine wordt genoemd. Het ubiquitinesysteem maakt gebruik van 3 enzymen, E1, E2 en E3, die samen zorgen voor de herkenning van eiwitten in de cel die moeten worden afgebroken. Een eiwit dat herkend wordt door E2 en E3, wordt vervolgens gekoppeld met het kleine eiwit ubiquitine. Als een eiwit gekoppeld wordt met meerdere, minstens vier, ubiquitine moleculen dan vormt deze polyubiquitineketen een herkenningssignaal voor een groot eiwitafbraak complex in de cel, het proteasoom. Het proteasoom is in staat om de gemerkte eiwitten gedeeltelijk of volledig af te breken, terwijl het ubiquitine zelf, telkens opnieuw kan worden gebruikt. Recent onderzoek heeft aangetoond dat het ubiquitinesysteem eveneens de verblijftijd van sommige receptoren aan het celoppervlak reguleert, op een dusdanige wijze dat cellen niet teveel en niet te weinig signalen van een bepaalde soort krijgen.

De groeihormoonreceptor is een voorbeeld van een eiwit waarvan de hoeveelheid aan het celoppervlak door het ubiquitinesysteem wordt gereguleerd. Het verwijderen van een receptor van de celwand kan echter niet door directe afbraak plaatsvinden. De receptor moet daartoe eerst naar het binnenste van de cel worden verplaatst via een proces dat internalisatie (endocytose) wordt genoemd (zie hoofdstuk 6, figuur 1, cijfer 3). In de cel wordt de receptor via het vroege endosoom (EE) en het late endosoom (LE) naar het lysosoom (L) getransporteerd, waar uiteindelijk de afbraak plaats vindt. Uit ons onderzoek blijkt dat het ubiquitinesysteem de internalisatie van de groeihormoonreceptor reguleert. Een speciaal motief in de aminozuurvolgorde van de groeihormoonreceptor (het UbE-motief) is belangrijk voor de herkenning van de receptor door het ubiquitinesysteem. In hoofdstuk 2 hebben we aangetoond dat de koppeling van ubiquitine aan de groeihormoonreceptor plaatsvindt aan het celoppervlak, in gespecialiseerde domeinen die belangrijk zijn voor internalisatie. Dat ook het proteasoom betrokken is bij het verwijderen van de groeihormoonreceptor van de celwand wordt duidelijk in hoofdstuk 3. Door gebruik te maken van stofjes die de werking van het proteasoom remmen, laten we zien dat een werkzaam proteasoom nodig is voor de internalisatie van de groeihormoonreceptor. De precieze rol van dit eiwitafbraakcomplex in het internalisatieproces hebben we nog niet vast kunnen stellen. De activiteit van het proteasoom is niet nodig voor de internalisatie van een groeihormoonreceptor met een verkorte cytosolische staart. Mogelijk wordt de cytosolische staart van de receptor door het proteasoom (gedeeltelijk) afgebroken waarna internalisatie en transport naar het lysosoom plaats kan vinden. Het is ook mogelijk dat de cytosolische staart van de receptor een ander eiwit bindt, dat eerst door het proteasoom gedegradeerd dient te worden, alvorens internalisatie kan plaats vinden. Toekomstig onderzoek zal het moleculaire mechanisme trachten te ontrafelen.

In hoofdstuk 4 laten we zien dat het ubiquitinesysteem eveneens betrokken is bij een sorteringsmechanisme in het vroege endosoom (zie hoofdstuk 6, figuur 1, cijfer 4). Na internalisatie, komen receptoren via blaasjestransport in het vroege endosoom terecht. Hier zorgt een sorteringsmechanisme ervoor dat sommige receptoren terugkeren naar de celwand om te worden hergebruikt (recycling), terwijl een ander type receptoren juist niet wordt hergebruikt maar naar het lysosoom wordt gestuurd voor afbraak. De groeihormoonreceptor wordt niet hergebruikt en het ubiquitinesysteem zorgt ervoor dat de receptor in het vroege endosoom in de route naar het lysosoom gesorteerd wordt.

De afbraak van de groeihormoonreceptor is een continu proces dat in het door ons gebruikte celsysteem niet afhankelijk is van de aanwezigheid van groeihormoon. In hoofdstuk 5 laten we zien dat 75% van de afbraak van de groeihormoonreceptor voor rekening komt van de ubiquitinesysteem-afhankelijke internalisatie. Een kwart van de receptoren wordt via andere routes afgebroken. Als in de groeihormoonreceptor het UbE-motief gewijzigd wordt, zodat de receptor niet meer herkend wordt door het ubiquitinesysteem, neemt de hoeveelheid receptoren aan het celoppervlak met ruim een factor twee toe. De herkenning van het UbE-motief in de groeihormoonreceptor door het ubiquitinesysteem, gevolgd door internalisatie en afbraak van de receptor in het lysosoom, vormt dus de basis voor de regulatie van de hoeveelheid receptoren aan het celoppervlak.

Nu blijkt in ziektes zoals sepsis, acidose, kanker en aids, het ubiquitinesysteem zo sterk geactiveerd te zijn dat de balans tussen eiwitsynthese en eiwitafbraak verstoord wordt. Uiteindelijk leidt deze balansverstoring tot een verlies aan vetdepots en afbraak van spiereiwitten, waardoor deze patiënten in het algemeen sterk vermageren. Verlies van eiwitmassa leidt tot cel- en weefselverval met bijbehorend functieverlies. Onze onderzoeksresultaten duiden erop dat het op hol geslagen ubiquitinesysteem niet alleen zorgt voor een verhoogde eiwitafbraak, maar mogelijk ook voor het verdwijnen van de groeihormoonreceptor van de celwand. Het gevolg is dat de cel de signalen van groeihormoon, die leiden tot aanmaak van spiereiwitten, niet meer kan ontvangen en doorgeven. Om de verstoorde balans te herstellen en om het groeihormoonsignaal tot eiwitsynthese weer door te geven, dient een manier te worden gevonden om het aantal groeihormoonreceptoren op de celwand te verhogen. De sleutel voor de oplossing van dat probleem ligt in de koppeling tussen het ubiquitinesysteem en de groeihormoonreceptor. Als we in staat zijn een stofje te vinden dat het ontstaan van deze koppeling verhindert, dan kunnen we mogelijk voorkomen dat in patiënten met een verhoogde activiteit van het ubiquitinesysteem, de groeihormoonreceptor van de celwand verdwijnt. Daardoor zal het groeihormoonsignaal kunnen worden doorgegeven en een betere balans ontstaan tussen afbraak en synthese van eiwitten, wat de lichamelijke aftakeling van een grote groep patiënten zal afremmen.

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Hoewel ik jarenlang heb gezegd dat ik nooit zou promoveren, is het er nu toch van gekomen. Ik heb dat niet in mijn eentje gedaan. Onderzoek doen is ‘teamwork'. Ik heb vele jaren in wisselende teams gewerkt en ben er van overtuigd dat de samenstelling van het team essentieel is voor onder meer kwaliteit en productiviteit en vooral voor het plezier in het werk. Het is juist het plezier in het verrichten van onderzoek dat mij heeft doen besluiten om toch te gaan promoveren. Vele mensen hebben in de loop van de jaren bijgedragen, aan inhoud, kwaliteit of het plezier en dit is de juiste plaats om een aantal van hen te bedanken.

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Curriculum vitae

Peter van Kerkhof werd geboren op 21 mei 1956 te Beneden-Leeuwen. Hij behaalde in 1973 het HAVO diploma aan het Pax Christi College te Druten. In datzelfde jaar startte hij de HBO-B opleiding, richting biochemie, aan de hogere laboratorium school te Oss. Hij deed zijn onderzoekstage bij de vakgroep Erfelijkheidsleer van de Landbouwhogeschool te Wageningen onder leiding van prof. dr. ir. J. Visser met als onderwerp "Karakterisering van het autolytisch enzymcomplex van Aspergillus nidulans". Het diploma werd behaald in mei 1977. Hierna heeft hij van november 1977 tot december 1978 zijn militaire dienstplicht vervuld aan de School voor Reserveofficieren en Kader Infanterie te Ermelo.

In januari 1979 trad hij als analist in dienst bij de vakgroep Histologie en Celbiologie van de Universiteit van Utrecht, op een door de Nederlandse organisatie voor zuiver-wetenschappelijk onderzoek (ZWO) gefinancierd project. Onder leiding van prof. dr. M.F. Kramer en drs. H.L. Smits werd gewerkt aan de analysering en karakterisering van maagslijmglycoproteinen. In juni 1981 kwam hij in dienst bij de vakgroep Histologie en Celbiologie en werkte onder leiding van prof. dr. C. Poort en dr. G.J. Strous binnen het VF (voorwaardelijke financiering)-programma Synthese en intracellulair transport van (glyco)proteinen. In 1985 bezocht hij het Dana Farber Cancer Institute, Harvard Medical School in Boston om onder supervisie van prof. dr. A.L. Schwartz een aantal biochemische technieken aan te leren.

Per 1 januari 1987 werd hij bevorderd tot hoofdanalist bij de vakgroep Celbiologie i.o. Vanaf deze tijd vervulde hij, naast het onderzoek, een aantal organisatorische en bestuurlijke taken in het vakgroepsbestuur, het dagelijks bestuur en de faculteitsraad. Om een poging te doen de financiële verslaglegging van vakgroep en faculteit te begrijpen behaalde hij in 1991 het praktijkdiploma boekhouden. Nadat moleculair biologische technieken hun intrede hadden gedaan bij de vakgroep Celbiologie, startte hij in 1993 de hogere laboratorium opleiding (HLO), richting microbiologie, aan de Hogeschool Utrecht. Het diploma werd behaald in juni 1994. In het kader van een toegekende subsidie uit het internationaliseringsproject voor analisten werkte hij in 1997 gedurende twee maanden bij het Department of Pediatrics, Washington University School of Medicine in St. Louis. In samenwerking met dr. G. Bu werd de fosforylering van het LDL-receptor related protein (LRP) bestudeerd. Hier werd duidelijk dat het uitvoeren van een promotieonderzoek een volgende stap in zijn carrière zou moeten zijn. In augustus 1998 begon hij, onder leiding van prof. dr. G.J. Strous, aan het in dit proefschrift beschreven onderzoek.

List of publications

Smits, H.L., van Kerkhof, P.J., and Kramer, M.F. (1982). Isolation and partial characterization of rat duodenal-gland (Brunner's-gland) mucus glycoprotein. Biochem. J. *203*, 779-785.

Smits, H.L., van Kerkhof, P.J., and Kramer, M.F. (1982). Composition and structure of rat Brunner's glands and gastric mucous glycoprotein. Adv. Exp. Med. Biol. *144*, 159-161.

Strous, G.J., Willemsen, R., van Kerkhof, P., Slot, J.W., Geuze, H.J., and Lodish, H.F. (1983). Vesicular stomatitis virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. J. Cell Biol. *97*, 1815-1822.

Strous, G.J., van Kerkhof, P., Willemsen, R., Geuze, H.J., and Berger, E.G. (1983). Transport and topology of galactosyltransferase in endomembranes of HeLacells. J. Cell Biol. *97*, 723-727.

Van Halbeek, H., Gerwig, G.J., Vliegenthart, J.F., Smits, H.L., van Kerkhof, P.J., and Kramer, M.F. (1983). Terminal $\alpha(1\rightarrow4)$-linked N-acetylglucosamine: a characteristic constituent of duodenal-gland mucous glycoproteins in rat and pig. A high-resolution ^{1}H-NMR study. Biochim. Biophys. Acta *747*, 107-116.

Strous, G.J., van Kerkhof, P., Willemsen, R., Slot, J.W., and Geuze, H.J. (1985). Effect of monensin on the metabolism, localization, and biosynthesis of N-and O-linked oligosaccharides of galactosyltransferase. Eur. J. Cell Biol. *36*, 256-262.

Strous, G.J., van Kerkhof, P., Fallon, R.J., and Schwartz, A.L. (1987). Golgi galactosyltransferase contains serine-linked phosphate. Eur. J. Biochem. *169*, 307-311.

Strous, G.J., van Kerkhof, P., Brok, R., Roth, J., and Brada, D. (1987). Glucosidase II, a protein of the endoplasmic reticulum with high mannose oligosaccharide chains and a rapid turnover. J. Biol. Chem. *262*, 3620-3625.

Strous, G.J., van Kerkhof, P., van Bokhoven, A., Schwartz, A.L., and de Pont, J.J. (1988). Effect of primaquine on the topology of Na,K-ATPase and the receptor for asialoglycoproteins. Prog. Clin. Biol. Res. *268B*, 437-442.

Strous, G.J., van Kerkhof, P., Dekker, J., and Schwartz, A.L. (1988). Metalloendoprotease inhibitors block protein synthesis, intracellular transport, and endocytosis in hepatoma cells. J. Biol. Chem. *263*, 18197-18204.

Strous, G.J., van Kerkhof, P., and Berger, E.G. (1988). In vitro biosynthesis of two human galactosyltransferase polypeptides. Biochem. Biophys. Res. Commun. *151*, 314-319.

Strous, G.J. and van Kerkhof, P. (1989). Release of soluble resident as well as secretory proteins from HepG2 cells by partial permeabilization of rough-endoplasmic-reticulum membranes. Biochem. J. *257*, 159-163.

Strous, G.J., Berger, E.G., van Kerkhof, P., Bosshart, H., Berger, B., and Geuze, H.J. (1991). Brefeldin A induces a microtubule-dependent fusion of galactosyltransferase-containing vesicles with the rough endoplasmic reticulum. Biol. Cell *71*, 25-31.

Strous, G.J., van Kerkhof, P., van Meer, G., Rijnboutt, S., and Stoorvogel, W. (1993). Differential effects of brefeldin A on transport of secretory and lysosomal proteins. J. Biol. Chem. *268*, 2341-2347.

Strous, G.J., van Kerkhof, P., Verheijen, C., Rossen, J.W., Liou, W., Slot, J.W., Roelen, C.A., and Schwartz, A.L. (1994). Expression of functional growth hormone receptor in a mouse L cell line infected with recombinant vaccinia virus. Exp. Cell Res. *211*, 353-359.

Strous, G.J., van Kerkhof, P., Govers, R., Ciechanover, A., Schwartz, A.L. (1996). The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. EMBO J. *15*, 3806-3812.

Strous, G.J., van Kerkhof, P., Govers, R., Rotwein, P., Schwartz, A.L. (1997). Growth hormone induced signal transduction depends on an intact ubiquitin system. J. Biol. Chem. *272*, 40-43.

Govers, R., van Kerkhof, P., Schwartz, A. L., Strous, G. J. (1997). Linkage of the ubiquitin-conjugating system and the endocytic pathway in ligand-induced internalization of the growth hormone receptor. EMBO J. *16*, 4851-4858.

Govers, R., van Kerkhof, P., Schwartz, A.L., Strous, G.J. (1998). Di-leucine-mediated internalization of ligand by a truncated growth hormone receptor is independent of the ubiquitin conjugation system. J. Biol. Chem. *273*, 16426-16433.

Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A.L., Strous, G.J. (1999). Identification of a novel ubiquitin conjugating motif, required for ligand-induced internalization of the growth hormone receptor. EMBO J. *18*, 28-36.

van Kerkhof, P., Govers, R., Alves dos Santos, C.M., Strous, G.J. (2000). Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. J. Biol. Chem. *275*, 1575-1580.

Li, Y., Marzolo, M., van Kerkhof, P., Strous, G.J., Bu, G. (2000). The YXXL motif but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. J. Biol. Chem. *275*, 17187-17194.

van Kerkhof, P., Sachse, M., Klumperman, J., Strous, G.J. (2001). Growth hormone receptor ubiquitination coincides with recruitment to clathrin-coated membrane domains. J. Biol. Chem. *276*, 3778-3784.

Li, Y., van Kerkhof, P., Marzolo, M., Strous, G.J., Bu, G. (2001). Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis. Mol. Cell. Biol. *21*, 1185-1195.

Alves dos Santos, C.M., van Kerkhof, P., Strous, G.J. (2001). The signal transduction of the growth hormone receptor is regulated by the ubiquitin/proteasome system and continues after endocytosis. J. Biol. Chem. *276*, 10839-10846.

van Kerkhof, P., Strous, G.J. (2001). The ubiquitin-proteasome pathway regulates lysosomal degradation of the growth hormone receptor and its ligand. Biochem. Soc. Transactions. *29 (4),* 488-493.

van Kerkhof, P., Alves dos Santos, C.M., Sachse, M., Klumperman, J., Bu, G., Strous, G.J. (2001). Proteasome inhibitors block a late step in lysosomal transport of selected membrane but not soluble proteins. Mol. Biol. Cell *12,* 2556-2566.

Sachse, M., van Kerkhof, P., Strous, G.J., Klumperman, J. (2001). The ubiquitin-dependent endocytosis motif is required for the incorporation of growth hormone receptor into clathrin-coated vesicles. J. Cell Sci., *in press*.

van Kerkhof, P., Smeets, M., Strous, G.J. (2001). The ubiquitin-proteasome pathway regulates growth hormone receptor availability. Submitted.