Ubiquitin System-Dependent Regulation of Growth Hormone Receptor Signal Transduction and Effects of Oxidative Stress

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Chapter I

General Introduction

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Introduction

The efficacy of the endocrine system relies on the specific interaction of a hormone to its receptor. In case of peptide hormones and cytokines, this involves complex interactions between proteins and multiple molecular recognition events. One of the best studied examples of these interactions is between growth hormone (GH) and its receptor (GHR). Understanding of the signaling mechanisms triggered by the binding of GH to GHR has rapidly advanced in the past several years. Several factors like stress, sleep, and exercise can enhance GH secretion in the blood, which then will bind cell-surface receptors (GHRs) on target tissues such as liver, muscle, adipose, and bone. This activates tightly regulated signaling cascades that will trigger several of the GH responses and promotes, at the same time, the GHR downregulation. The ubiquitin-proteasome system plays also an important role in the GHR downregulation. GH or GHR defects, like mutations or deletions can have important effects in GHR's signal transduction or downregulation, which can lead to severe deficiencies and syndromes. GH has long been used to treat children with short stature and may increase protein synthesis and prevent muscle wasting in patients with burns or AIDS. The GHR and it hormone are of basic importance for regulation of metabolism in man, and therefore its cell biology must be carefully and precisely regulated.

The cytokine/hematopoietin receptor superfamily

The GHR is a single-transmembrane polypeptide that belongs to the class I of the cytokine receptor superfamily (1). All members of this family, which include receptors for prolactin (PRL), erythropoietin (Epo), thrombopoietin, leptin, ciliary neurotropic factor (CNTF) leukemia inhibitory factor (LIF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), and several interleukins, share homologous structural motifs (2, 3). Receptors for interferon (IFN) α/β, IFNγ, and IL-10 are more distantly related and considered class II receptors in the family (4). The class I cytokine receptors span the membrane once and contain an extracellular region, a single hydrophobic transmembrane domain of 24 amino acids, and an intracellular region. In their extracellular ligand binding domain, four conserved cysteine residues are found in the amino-terminal part and a conserved WSXWS (Trp-Ser-X-Trp-Ser) motif is present in the C-terminal part, predicted to function as a ligand interaction site (5). In the GHR, this motif is altered in amino acids Tyr-Gly-Glu-Phe-Ser (YGEFS). It does not contact ligand, but upon a mutation of the last serine, both binding affinity and trafficking of the receptor are impaired (6). Because it does not make direct contact with ligand, the YGEFS motif is likely to serve a structural role critical for ligand binding (7). In the intracellular domains of cytokine receptors, no consensus catalytic site was identified, and limited amino acid homology was only recognized in two motifs: Box-1 and Box-2, located in the cytoplasmic membrane-proximal region of

several members of the cytokine family (8), and known to be involved in signal transduction (8, 9). Box-1, a proline-rich motif present in all members, consists of a Pro-Xaa-Pro sequence and a preceding cluster of hydrophobic amino acids. Another motif, Box-2, is present in most of these receptors and is only 50% conserved in the members of the receptor family. It is characterized as a cluster of hydrophobic amino acids, is followed by negatively charged residues, and ends with one or two basic amino acids. Another notable feature of this family of receptors is their multi-component nature. This suggests that some receptors could share components, providing a potential explanation for some of the remarkable functional redundancy observed among several cytokines.

The Growth Hormone Receptor (GHR) structure

Physically, GHR is characterized by a Mr of ~ 130,000 (10). Based on amino acid sequence, the calculated molecular mass of the GHR is 70 kDa, but posttranslational modifications such as glycosylation and ubiquitination are responsible for the higher molecular mass experimentally observed. The GHR was initially cloned from rabbit and human cDNA libraries and subsequently from other species, presenting amino acid sequence homologies of ~70% between the different species (11). The rabbit GHR contains a total of 620 amino acid residues, of which 246 are part of the extracellular domain, 24 of the transmembrane domain, and 350 of the intracellular domain. The GHR extracellular domain consists of two domains (amino acids 1-123 and amino acids 128-238) that are linked by four residues (amino acids 124-127) (7). Each domain contains seven β strands arranged to form a sandwich of two antiparallel β sheets. The N-terminal domain contains six cysteines that form three disulfide bonds between Cys 38-48, Cys 83-94, and Cys 108-122 and one free cysteine (7, 12). The first of these disulfide bonds is essential for ligand binding (13). Hydrogen bonds between Arg 43 and Glu 169, and a salt bridge between Arg 39 and Asp 123 further stabilize the GHR (14). Five potential N-linked glycosylation sites are conserved in the extracellular domain of the GHR in species sequenced to date, several of which are glycosylated (15). The presence of alternatively spliced mRNAs potentially encoding for short forms of the GHR has been reported (16, 17). Such putative forms, which have not yet been demonstrated to be readily translated in vivo, seem to be degraded more easily than the long form of the receptor. In addition to the membrane-bound form of GHR, a soluble circulating form of the receptor, named growth hormone binding protein (GHBP), has been characterized. GHBP is identical to the extracellular domain of the GHR and depending on the species, different mechanisms are used to generate GHBP. In rat and mouse, GHBP is encoded by a specific mRNA originated from alternatively splicing, and contains a short hydrophylic C-terminal extension that is not found in the membrane receptor (18, 19). Normally, the GHBP is generated by proteolytic cleavage at the cell-surface, a process also known as shedding, of the membrane-

bound form of the receptor by a recently identified metalloprotease called tumor necrosis factor (TNF)-α-converting enzyme (TACE or ADAM-17) (20). Diverse biological functions have been attributed to the GHBP. In plasma, complex formation of GHBP-GH creates a circulating GH reservoir, protects GH from degradation and excretion, prolongs its half-life, and may enhance its bioactivity in vivo through these mechanisms (21, 22). In tissues, GHBP acts in a dominant negative manner by competing with GHRs for ligand and by forming unproductive GHR-GHBP heterodimers that are unable to signal (23). Direct and indirect evidence, particulary in the liver, have suggested the GHBP as an useful tool of measuring GHR abundance at plasma levels (24).
Possible GHR isoforms described above could be responsible for generation of a large amount of GHBP. Such isoforms may act as a dominant negative to the long form of GHR, suggesting that their expression could then modulate signaling by the full-length receptor. Abnormal expression of one of these short forms, as well as mutations in the GHR (deletion, frameshift, nonsense mutation or amino acid substitution) have been shown in patients with Laron sindrome.

GH

Growth hormone (GH), also termed somatotropin or somatotrope hormone, is a polypeptide hormone essentially secreted by the pituitary gland that exerts its effects via cell-surface specific receptors located at the surface of target issues (25). GH has long been known to mainly promote postnatal longitudinal body growth and differentiation of muscle, bone, and cartilage cells, including organ size and longitudinal bone growth (26).
Through the interaction with the GHR, GH regulates the lipid, carbohydrate, nitrogen and mineral metabolism within a cell (27, 28). For example, recombinant porcine GH administered to pigs and human GH administered to older men increases lean body mass and decreases fat (29, 30).
GH induces two opposite actions on carbohydrate metabolism. First, the insulin-like effect which last approximately 2 hours and involves lipogenesis and enhanced glucose and amino acid metabolism (27). Glucose is taken into the cell and stored as glycogen. Second, the anti-insulin effect which occurs much later (after 3 hours) and includes lipolysis (production of free fatty acids), hyperglycemia, and hyperinsulinemia. The anabolic effects of GH lead to an increase in protein synthesis ultimately resulting in an increase in lean muscle mass (31). GH plays an integral role in the maintenance of the immune system. It is important for lymphocyte function and required for a critical period of fetal bovine immune system development (32). Through direct actions on the brain, GH may also modulate emotion, stress response, and behaviour (33).
Many of the actions of GH are mediated by the activation of insulin-like growth factor one (IGF-1). GH causes differentiation of precursor cells and also can stimulate

IGF-1 secretion. The secreted IGF-1 exhibits a mitogenic effect on the differentiated cells to cause clonal expansion of cell numbers. IGF-1 is produced by the liver, bone and other types of tissues in response to GH and acts in a paracrine and endocrine fashion (27). There is growing evidence that GH and IGF-1 are involved in heart development and hypertrophy (34). Recombinant human GH (rGH) was shown to increase myocardial mass and improve myocardial energy metabolism and hemodynamics (34).

Hypersecretion of the hormone can lead to giantism and acromegaly in adults (35). This condition causes enlarged bones, especially in the face, as well as oversized organs. Alternatively hyposecretion of GH can lead to dwarfism. Body-fat reduction, one of the GH-induced actions, is clinically important in replacement therapy for adult GH deficient patients with obesity. Although this action can be attributed to hydrolysis of stored triglycerides by activation of hormone-sensitive lipase (HSL), details on the molecular mechanism of GH-induced lipolysis stimulation are still poorly understood (36). The major physiological component of pituitary gland secreted GH (about 75%) is a single chain polypeptide of 191 amino acids and a molecular mass of 22 kDa. At least four other monomeric GH isoforms, with molecular masses 27, 20, 17 and 5 kDa, have been detected in the circulation to date, and it has been reported that they might be partly responsible for short stature and growth retardation in children (37). These non-22-kDa isoforms may be weak agonists or even antagonists of the GHR binding, resulting in altered receptor homodimerization and activation depending on the relative affinities for binding sites 1 and 2 (see below) to the GHR (38).

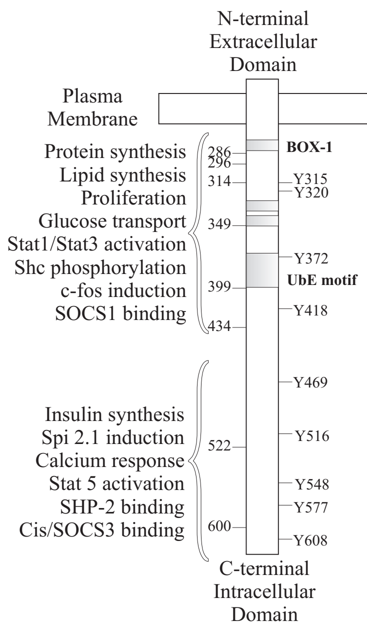

Figure 1. Squematic representation of the GHR.

Regulation of GHR expression

Development (ontogeny), hormonal presence, nutritional status, and tissue/cell specific control taken place at the transcriptional or translational level, regulate GHR gene expression. Chronic GH treatment increases GH binding in hepatic tissues of pig, rat and sheep (39, 40). GH upregulates the number of GH binding sites at the cell-surface but, at the same time, initiates GHR downregulation (41, 42, 43).
Mal-nutrition and fasting lead to a state of GH resistance characterized by increased circulating levels of GH and decreased IGF-1 concentrations in mammals (44). These hormonal changes are accompanied by a decrease in GHRs in hepatic membranes (45). Glucose starvation results in a significant reduction (up to 45%) of the expression of GHR in the presence of GH. Removal of arginine, proline, tryptophan, or valine resulted in only slight decreases in GHR mRNA. Energy, in the form of glucose, appears to control GHR expression (46).

Signaling Mechanisms of the GHR

GH-GHR2 dimer

Studies on the crystallographic structure of GH-GHR complexes revealed a trimeric complex of two receptors and a single molecule of hormone (7). Analysis of the complex revealed two binding sites on hGH on opposite sides with slightly different binding affinities (47). The ligand binding domain and dimerization interface at the extracellular part of the GHR lie on its cystein pairs and YGEFS motif. The GH binding to the two receptors is considered the first step in the action of GH. This process is initiated with GH binding to one molecule of receptor through its site 1, a functional epitope of 11 amino acids, followed by association of this complex to a second receptor molecule through GH's site 2, a 31 amino acid region that stabilizes and, together with a ~500 Å contact region between the two extracellular domains of GHR, defines the binding of the second GHR (reviewed in: 14). The stoichiometry of a single GH binding two GHRs and the fact that site 1 and 2 of GH are largely composed of the same residues suggest that one GHR cannot bind more than one GH molecule. Receptor dimerization is crucial for signal transduction since a hGH antagonist, G120R, still being able to bind to the first GHR via its site 1 but defective in its site 2 to induce dimerization, cannot transduce signal, being however internalized as efficiently as the wild type ligand (48, 49). Such compounds can be used to compete with the natural ligand to transduce signal and be designed for therapeutic use (50).

GHR dimerization & JAK2 activation

Although the GHR and other cytokine receptors do not possess intrinsic tyrosine kinase activity, GH binding to its receptor results in rapid tyrosine phosphorylation of multiple cellular proteins due to the activation of GHR-associated tyrosine kina-

ses of the JAK family. The ability to associate with and activate tyrosine kinases is essential to propagate many if not most intracellular signals (e.g. the proliferation, gene induction, and activation-signaling molecules like phosphatidylinositol 3 (PI3)-kinase, MAP kinase and ras). The JAK kinases represent a distinct family of soluble tyrosine kinases that have been strongly implicated in the signal transduction of many members of the cytokine family. The four members of the family, which include Tyrk2, JAK1, JAK2, and JAK3, have molecular weights ranging from 125-135 kDa (for review see Ref. 51). Among known members of the JAK family, JAK2 is the main kinase activated by the GH-GHR complex and considered the initiating step in GHR signal transduction (10). Activation of JAK2 by GH was shown in various cell systems and in vivo in liver, heart, kidney, and adipose tissue (52). Some activation of JAK1 was also described (53) and another high molecular weight JAK2 antigenically related protein was reported to be phosphorylated upon GH stimulation (54). A direct comparison of the four JAK family members revealed the presence of seven highly conserved domains (JH1-JH7) and a sequence identity of 35-45% (55). The family members consist of two kinase-related domains JH1 and JH2 and do not contain any SH2 or SH3 domains. The importance and function of the other five domains remain poorly understood. Only the most carboxyl-terminal domain is believed to be functional, while the N-terminal region is thought to be involved in GHR association, as suggested by the activity of deletion mutants of the tyrosine kinase. Deletion of the kinase domain eliminated functional coupling but not physical association of JAK2 with the GHR (56).

Studies using mutated and truncated GHRs have indicated that box-1, the proline-rich region in the cytoplasmic domain of the GHR, is necessary for GH-dependent association and activation of JAK2 with the GHR, (57, 58, 59). No specific amino acid within box-1 is essential for the association between the receptor and JAK2. Mutation of each individual proline residue in box-1 or the simultaneous mutation of the first two prolines did not impair the kinase association. However, a specific secondary structure of the receptor is required, in which simultaneous mutation of the last two prolines or of the three hydrophobic residues (isoleucine; leucine and valine into threonine) abolishes the capacity of the receptor to interact with and to activate JAK2 (60). The box-1 sequence of the GHR is very similar to proline-rich SH3 domains. However, as mentioned above, no SH3 domain has been identified in JAK2, and the existence of an SH3-containing adaptor protein could mediate or facilitate the association between cytokine receptors and JAK kinases. The first 46 residues of the GHR containing the box-1 are sufficient to induce some JAK2 activation, but maximal activity of this kinase requires downstream residues in the half-proximal transmembrane part of the GHR cytoplasmic domain. This region appears to stabilize the interaction between the receptor and JAK2.

Normally, JAK kinases are thought to be catalytically inactive in resting cells being however associated with the cytoplasmic domains of receptor chains. Upon ligand-

stimulation, the GHR becomes dimerized, increasing the affinity of each GHR for JAK2. This brings two JAK2 molecules into close proximity allowing each JAK2 to transphosphorylate one or more tyrosines within the kinase domain of the paired JAK2, thereby activating the JAK2 kinase. It is important to note that, while multiple ligands activate the same JAK, they do not necessarily activate it to the same extent and with the same time course in the same cell line.

GHR signaling pathways

As mentioned above, upon GH binding to its receptor, activation of JAK2 is rapid and transient. Once activated, JAK2 phosphorylates the GHR on multiple tyrosine residues providing docking sites for other signaling molecules (61). JAK2 transient phosphorylation can reach a maximal activity at 5-20 min depending on cellular types and return to basal levels after 60 min of stimulation. JAK2 activation appears to be sufficient to mediate some of the effects of GH, such as stimulation of cell proliferation and the activation of signaling molecules involved in this effect (57). JAK2 contains 48 tyrosines, all which might become phosphorylated upon GH, suggesting that this kinase can interact with multiple signaling molecules (62). Among the direct substrates of JAK2 are several docking proteins involved in the Ras/MAP kinase pathway, the STAT proteins (signal transducers and activators of transcription), as well as the insulin receptor substrate proteins IRS-1 and IRS-2, which initiate the PI-3 kinase pathway (reviewed in: 63). Another protein identified as a JAK2 substrate was SH2-Bβ which function is still not established (64). So far, only the GH-dependent calcium transport appears to involve a pathway that is independent of JAK2 activation (65). The GHR has several conserved tyrosine residues in its intracellular domain that can become phosphorylated and appear to play a role in different actions of GHR. Other GH effects like GH-dependent expression of the insulin gene or the serine protease inhibitor (Spi) 2.1 gene require additional sequences in the GHR cytoplasmic domain (66, 67). In a more distal C-terminal part of the GHR cytoplasmic domain, the tyrosine phosphorylation of residues Y487, Y534, Y566 or Y627 of pig GHR is required for GH-induced tyrosine phosphorylation of STAT5 (68), while Y333 and/or Y338 in rat GHR appears to be required for GH-stimulated lipid and protein synthesis (69) and maximal activation of STATs 1, 3 and 5 (53).

STATs

STAT proteins are a family of transcription factors that couple ligand binding to cellular receptors with the activation of gene transcription (70). Currently, the sequence of seven genes from mammals that encode STAT family members have been reported (reviewed in: 71, 72). This family contains many conserved features: (a) the N-terminal 140 amino acids are conserved; (b) the presence of a leucine-rich heptad repeat region located approximately 200 residues from the N-terminus (dele-

tion of the region results in STAT inactivation); (c) an SH2 domain important for binding to phosphorylated tyrosine residues; (d) a tyrosine residue located at the C-terminus that is a substrate for JAK2; and (e) a serine phosphorylation site (73). GH was found to be able to activate STAT1, STAT3 and both isoforms of STAT5, STAT5a and STAT5b (53). STAT1 was initially identified as a transcription factor induced by interferons (74), STAT3/acute phase response factor (APRF) identified in IL-6 and LIF signaling (75, 76, 77), and STAT5 was first cloned as a prolactin-responsive factor, initially named mammary gland factor (78). Upon JAK2 tyrosine phosphorylation, cytoplasmic STAT proteins are recruited through their SH2 domain in the complex GHR/JAK2 kinase and are subsequently phosphorylated by JAK2 on their conserved C-terminal tyrosine. Activated STATs homo- or heterodimerize with other STATs and/or non STAT proteins, presumably by interactions involving the SH2 and SH3 domains of the STAT proteins, translocate to the nucleus, bind DNA and activate transcription of target genes (79). STAT1 and STAT3 are phosphorylated in response to GH and bind to the c-sis-inducible element (SIE) of the c-fos promoter/enhancer (80, 81). Similarly, GH promotes the binding of STAT5 to the GH-responsive element 2 (GHRE-2) in the promoter region of the Spi 2.1 gene (67, 82). Activation of the different STATs by GH differs among tissues and cell lines. In 3T3-F442A fibroblasts, in liver from hypophylsectomized rats treated with GH and CHO cells expressing recombinant GHR, it was shown that GH induced tyrosine phosphorylation of both STAT1, 2 and 5 (53, 83). High levels of unphosphorylated STAT1 and STAT3 were found in the liver in both male and female rats, whereas tyrosine phosphorylated STAT5b was present at a high steady-state level only in male nuclear extracts (84). In CHO cells transfected with rabbit GHR, GH induced the formation of complexes consisting of STAT5a and the MAP kinase ERK2, and STAT5a and STAT5b association with the adaptor protein SHC (85).

The regions of the GHR required for activation of the different STATs has been mapped. STAT1, STAT3 and STAT5 all require the membrane proximal part of the GHR including box-1, and therefore JAK2 activation by GH in order to become phosphorylated and activated (86, 80, 81, 53). Furthermore, STAT5 activation in response to GH most likely results as a consequence of STAT5 binding to multiple binding sites within the GHR, like phosphorylated tyrosines that are present within both the C-terminal half and N-terminal half of the cytoplasmic domain of the GHR (87, 61, 88). In pGHR, it was found that Y322, Y487, Y534, Y566, and Y627 are phosphorylated in response to GH (61). In addition, tyrosine residues in the C-terminal region of the GHR transfected in CHO cells are necessary for serine phosphorylation of the STAT5a isoform, which then associates with other proteins, like ERK2, and both STAT5 isoforms interact with the serine-threonine kinase Akt-1, indicating the importance of serine phosphorylation cascades in GH signaling (85).

MAP kinases

The main signaling pathways stimulated by GH are shown in Fig 1. Two such proteins that are activated by GH are the MAP kinases ERK1 and ERK2 (89, 90, 91). MAP kinases are a family of the serine/threonine/tyrosine kinases that mediate both cellular growth and differentiation upon stimulation by many growth factors and hormones (reviewed in: 92). One pathway leading to MAP kinase activation involves the SHC tyrosine phosphorylation (87), followed by the sequential recruitment of Grb2, Son-of-sevenless (Sos), Ras, Raf, and MAP kinase kinase (MEK) (93, 94). The binding of SHC through its SH2 domain to phosphorylated residues in both JAK2 and the GHR is the first step in GH-mediated MAPK activation (87). GH rapidly and transiently stimulates tyrosine phosphorylation of the 46-, 52-, and 66-kDa known isoforms of SHC (87). It is thought that JAK2 phosphorylates SHC, as GHR mutants that fail to bind and activate JAK2 are unable to phosphorylate SHC and activate ERK1 and ERK2 (87, 59). Active SHC then interacts with and activates growth factor receptor bound to Grb2. Grb2 can then activate Sos, which in turn activates RAS, RAF, MEK and MAPK (94). MAPK activation by GH may be mediated via activation of the epidermal growth factor (EGF) receptor (95). It has been reported that EGFR was phosphorylated by JAK2 and may act as a docking protein for Grb2. This finding is one example of cross-talk between membrane receptors of different families.

Insulin Receptor Substrates-1 and -2 (IRS-1 and IRS-2) and Phosphatidylinositol 3-kinase (PI 3-K)

Upon GH stimulation, rapid insulin-like effects (read above, GH) like increased amino acid transport, glucose transport and lipogenesis occur (28). The finding that GH can interact with IRS-1 and induce its phosphorylation was observed in rat adipocytes, in 3T3-F442A fibroblasts or in CHO cells expressing GHR (96). IRS-2 was also reported to be associated to GHR complexes (51). IRSs were originally identified as signaling molecules for insulin and the closely related IGF. IRS-1 and IRS-2 are large cytosolic proteins (160-180 kDa) that appear to serve as mobile adapter proteins. They are phosphorylated on multiple tyrosines in response to insulin stimulation and form high-affinity binding sites for signaling molecules like the p85 regulatory subunit of phosphatidylinositol kinase (PI 3-kinase, which is activated upon p85 binding to IRS proteins) (97, 51, 98), the SH2 domain-containing tyrosine phosphatase two (SHP2) (51, 99) the Src family kinase fyn, Nck, and Grb2 (100, 101, 102).

The nature of the GH-stimulated interaction with IRS-1 is still not clearly understood. Studies using truncated and mutated GHRs expressed in CHO cells showed that the regions of the receptor required for tyrosine phosphorylation of IRS-1/IRS-2 were the same as that required for JAK2 activation (51). Tyrosine phosphorylation of IRS-1 and IRS-2 is dependent on JAK2 activation and interaction of the

GHR-JAK2 complex with IRS-1 and IRS-2 occurs through activated JAK2 either directly or via an intermediary protein. GH promotes the binding of the 85-kDa regulatory subunit of PI 3-kinase to IRS-1 and IRS-2 (51). PI 3-kinase is implicated in several pathways, including DNA synthesis, glucose uptake, cell cycle regulation via p70 ribosomal subunit kinase (rsk) activation (103), and inhibition of apoptosis via Akt serine threonine kinase (104). Most cytokine receptors induce the recruitment of PI 3-kinase in the activated cytokine receptor complex, the interaction being direct or indirect. In the case of the GHR, PI 3-kinase could associate with either the receptor (105), JAK2, or IRS-1 and IRS-2. However, PI 3-kinase is activated but not tyrosine phosphorylated under GH stimulation. The role of PI 3-kinase in GHR signaling could partially explain the insulin-like effects of GH as wortmannin, a PI 3-kinase inhibitor, blocks the ability of GH to stimulate lipid synthesis (106).

· PLC, PKC, Ca^{2+} pathways

GH has been found to activate protein kinase C (PKC) through the phospholipase C (PLC) signal transduction pathway (105). Activation of PKC by GH is thought to stimulate lipogenesis (107), induce c-fos expression (108), increase intracellular Ca^{2+} concentration (109), and stimulate binding of nuclear protein to C/EBP oligonucleotide (110). It is still not clear whether PKC is involved in the activation of MAPKs and p90RSK (89, 90, 91). PKC is a family member of serine/threonine

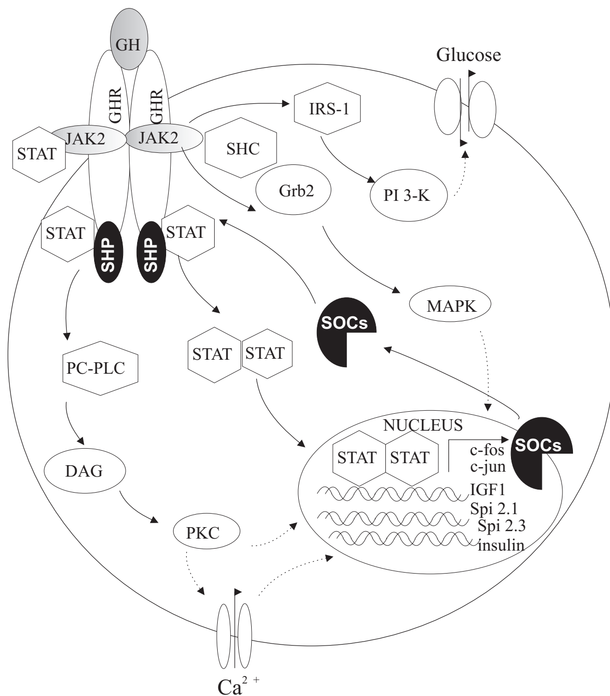

Figure 2. GHR Signal transduction pathways.
Black images represent negative regulators of GHR signaling

kinases consisting of at least 12 isoforms that activate several proteins, and important regulators of cellular growth, differentiation and metabolism (111, 112). For most of the PKC isoforms, activation and translocation from the cytosol to the cellular membrane is mediated by the second messenger Ca^{2+} and/or 1,2-diacylglycerol (DAG). GH transiently induces activation of DAG, an activator of PKC, in multiple cell types (113, 114), that can then bind, recruit, and stimulate PKC (115). Activation of the α-, β-, and γ-isoforms requires Ca^{2+} while the ε-, δ-, ν-, ζ-, and θ-isoforms are Ca^{2+} independent. The inositol 1,4,5-trisphosphate (IP3) pathway that generates both Ca^{2+} and DAG can activate PKC isoforms distinct from the isoforms activated following phospholipase C-mediated or phospholipase D-mediated hydrolysis of phosphatidylcholine with generation of just DAG (reviewed in: 111, 112). Phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, byproducts of PI 3-kinase, have been shown to activate the Ca^{2+} independent PKC isoforms ε-, δ-, and ν- (116). The findings that GH induces translocation of the ε-isoform of PKC from the cytoplasm to the plasma membrane in 3T3-F442A fibroblasts (117) and that wortmannin inhibits GH-dependent DAG formation in rat adipocytes (118) raise the possibility that in some cell types GH activation of PKC may involve IRS-1, IRS2, and PI 3-kinase. Ca^{2+} increase by GH seems to be dependent on L-type calcium channel activation by a mechanism including phospholipid hydrolysis and PKC activation (as mentioned above). Ca^{2+} seems to be important for activation of some GH-induced genes such as the Spi2.1 gene and the effects of GH on metabolism in adipocytes (119, 65). Ca^{2+} activation is until now the only pathway activated by GH that does not require Box-1 but only the C-terminal part of the GHR (65).

Negative Regulators of GH Signaling

Phosphatases

Pre-incubation with pervanadate, which inhibits phosphatase activities, resulted in a prolonged JAK2 and STAT5 tyrosine phosphorylation (120), indicating that phosphatases are involved in the inactivation process. Upon truncation of the C-terminal region of the GHR tail, JAK2 phosphorylation was sustained and STAT3 and IRS-1 activation enhanced (121). This suggests that a phosphatase may interact with the C-terminal cytoplasmic domain of the GHR. The tyrosine phosphatase SHP-1 (also known as HCP, SH-PTP-1, and PTP1C) was found to interact with JAK2 and dephosphorylate the kinase (122, 123). SHP-1 is a SH2 domain-containing tyrosine phosphatase that is expressed primarily in haematopoietic cells. However, since SHP-1 does not associate with the GHR, and as downregulation of JAK2 still occurs in the liver of knockout SHP-1 mice (122), it is likely that another mechanism contributes to the downregulation of JAK2 activation. SHP-2, another phosphatase, was found to associate with the GHR (124) and bind to JAK2,

but no dephosphorylation of JAK2 by SHP-2 was demonstrated (125). On the contrary, SHP-2 was found to positively regulate GHR signaling (124). Recently, the transmembrane PTPase CD45, a key regulator of antigen receptor signaling in T and B cells (126), was found to supress JAK kinases and negatively regulate cytokine receptor signaling (127). Targeted disruption of the cd45 gene lead to enhanced cytokine and interferon-receptor-mediated activation of JAKs and STAT proteins. In vitro, CD45 directly dephosphorylated and bound JAKs and was shown to negatively regulate interleukin-3-mediated cellular proliferation, erythropoietin-dependent haematopoieisis and antiviral responses in vitro and in vivo (127).
Kinase inhibitors such as H7, which inhibits serine threonine kinases, resulted in sustained JAK2/STAT activation, suggesting that besides protein dephosphorylation, protein phosphorylation is also required. Furthermore, both inhibition of protein synthesis with cycloheximide and inhibition of protein degradation with proteasome inhibitors such as MG132 induced prolongation of the signal (128, 120). Several processes are probably involved in GHR signal downregulation that require both activation of phosphatases, activation of kinases, protein degradation and protein biosynthesis. Therefore, other molecules that could bind to the C-terminal region of the GHR would contribute to signal downregulation.

SOCS proteins

A recent identified new family of negative regulators of cytokine signaling is the suppressor of cytokine signaling (SOCS). The SOCS family, including the immediate early gene CIS (cytokine inhibitor of signaling), comprises of at least eight proteins (SOCS-1 to SOCS-7, CIS) with similar structures (129, 130, 131, 132, 133). The SOCS proteins contain a variable region at the N-terminus, a centrally located SH2 domain mediating binding to phosphotyrosine residues in target proteins, and a region of homology at the C-terminus termed the SOCS box (130). The SOCS box is a region of approximately 40 amino acids which is unrelated to the sequence of other motifs and is of unknown function (130, 132). Searches on DNA databases for sequences homologous to SOCS box have identified 12 proteins containing this motif (132, 134). Besides the SOCS proteins, three new families of proteins containing either WD-40 repeats, SPRY domains or ankyrin repeats N-terminal to the SOCS box were described (132). The function of these proteins is still unknown. Furthermore, a class of small GTPases contains a SOCS box motif (132). Expression of these proteins exhibit unique tissue-specific and time-dependent responses to a broad range of cytokines, with different stimulation kinetics between the different SOCS genes, that is mediated, at least in part, by STAT proteins. Expression of the CIS gene is modulated by STAT5 and STAT3 is important for SOCS-1 expression (135, 136), forming a classical feedback loop that regulates cytokine signal transduction. CIS was found to bind to the tyrosine phosphorylated Epo and IL-3 receptors and block STAT5 phosphorylation and downstream STAT5-

dependent transcriptional responses and, in this way, to supress proliferation of hematopoietic cells in response to IL-3 (137, 135). In contrast, SOCS-1 binds to the phosphorylated JH1 domain of JAK2 and this association is likely to be mediated, at least in part, by its SH2 domain, although this interaction alone is insufficient for inhibition of a biological response (138). SOCS/CIS mRNA expression can be induced in liver cells in response to a pulse of GH (139, 140), and both SOCS-1 and SOCS-3 were found to inhibit GH-induced STAT5-dependent transcriptional responses in transfected cells (139, 141). Expression of other SOCS genes, SOCS-2 and CIS, were also up-regulated by GH, although to a lesser extent than SOCS-3 and with differing kinetics (139). SOCS/CIS proteins inhibit GH-stimulated tyrosine phosphorylation of STAT5b and STAT5b-dependent gene transcription by three distinct mechanisms, distinguished by their targets within the GHR-JAK2 signaling complex. GHR tyrosine residues 333 and 338, required for GH-stimulated lipogenesis and protein synthesis (69), are phosphorylated in response to GH stimulation (142) but are not obligatory for GH-stimulated STAT5b activation (69), and are known to play a key role in mediating the inhibitory effects of SOCS-3 on GH signaling (143). The binding of SOCS-3 to GHR tyrosines 333/338 could inhibit JAK2 signaling to STAT5 by interfering with JAK2 binding to the adjacent GHR box-1 region (residues 298-311), thereby raising the Kd of the receptor-kinase complex (143). SOCS-1 acts at the level of JAK2 tyrosine kinase, as JAK2 tyrosine phosphorylation is strongly inhibited by SOCS-1 protein (143). SOCS-1 inhibition of JAK2 kinase activity requires binding interactions between SOCS-1's SH2 domain and tyrosine 1007 within JAK2's kinase activation loop (144). The inhibition of JAK2 activity additionally requires a 13-residue "kinase inhibitory peptide" that is immediately NH2-terminal to SOCS-1's extended SH2 domain, and is proposed to function as an inhibitory, non-phosphorylable pseudosubstrate of JAK2 (144, 145). Furthermore, SOCS-1 can bind to the 80 COOH-terminal cytoplasmic residues of GHR, even in the absence of tyrosine phosphorylation, suggesting that it may be associated with unstimulated receptor molecules. Possibly, the binding of SOCS-1 to these GHR sequences may serve to correctly orient SOCS-1 or perhaps increase its affinity for the receptor-kinase signaling complex (143). In the case of CIS and SOCS-2, but not SOCS-3, GHR residues 540-620 were sufficient for SOCS/CIS binding to the receptor (143). GH stimulates phosphorylation of two tyrosines within this region (rat GHR tyr 566 and 627; hGHR tyr 548 and 609), both of which provide functional docking sites for STAT5 (88). The inhibition by CIS or SOCS-2 of GHR-JAK2 signaling to STAT5 could result from direct competition between STAT5 and either CIS or SOCS-2 for tyrosine-phosphorylated binding sites on GHR. Alternatively, the inhibition of GH signaling by CIS or SOCS-2 could involve SOCS/CIS protein ubiquitination followed by degradation of the the SOCS/CIS-bound GHR-JAK2 complex. It has been reported that the SOCS box binds elongins B and C (134). The elongin BC complex has been shown to bind

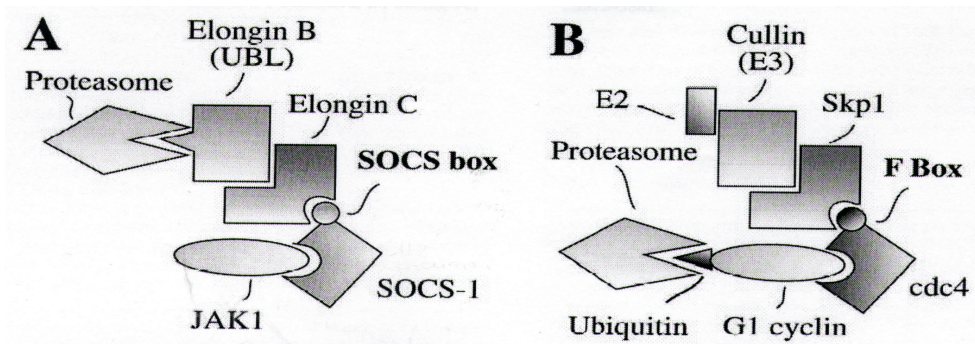

Figure 3. Model of SOCS action
Model of the interaction of SOCS-box containing proteins with elongins C and B (A) and comparison with the PULC assembled by F box-containg proteins (B). Black images represent negative regulators of GHR signaling

elongin A to form an active transcriptional elongation complex or to the von Hippel Lindau (VHL) tumor suppressor protein (146, 147). The VHL/elongin B-C (VCB) complex also contains a putative E3 ubiquitin ligase (Cullin-2) that may target VHL-binding proteins to destruction by the proteasome (148). The interaction sites on elongin A and VHL that interact with elongin C are homologous to the N-terminal half of SOCS box (147), suggesting that the primary interaction is between the SOCS box and elongin C (149). Interestingly, elongin B contains an ubiquitin-like (UBL) sequence at its N-terminus (150), suggesting a model for the action of the SOCS proteins. First, the N-terminal and SH2 domains of SOCS-1 and SOCS-3, at least, are required for recognition and binding to activated JAKs. On the other hand, the SOCS box brings into this complex elongins B and C. The model suggests that, either through direct interactions of the elongin B UBL domain with the proteasome or through associated Cullin-2-induced ubiquitination of substrates and subsequent proteosomal association, JAK2 and the associated SOCS proteins may be destroyed (149). In favor to this model, it was demonstrated that CIS and SOCS-1 are degraded in a proteasome-dependent manner (149, 151). SOCS-1 was shown to program the hematopoietic specific guanine nucleotide exchange factor, VAV, for ubiquitin /proteasome-mediated degradation by acting as a substrate-specific recognition component of a VCB-like ubiquitin ligase complex (151). Furthermore, CIS was shown to become ubiquitinated and its degradation was inhibited in presence of proteasome inhibitors upon EPOR stimulation (152). It was further shown that CIS inhibits GHR-JAK2 signaling by two distinct mechanisms: (1) a partial inhibition that is decreased at elevated STAT5b levels and may involve competition between CIS and STAT5b for common GHR cytoplasmic tail phosphotyrosine-binding sites; and by a time-dependent inhibition, not seen with SOCS-3, that involves proteasome action (153); (2) GH was shown to induce degradation of CIS, but not SOCS-

3, and the proteasome inhibitor MG132 blocked CIS degradation as well as it inhibitory action on STAT5b signaling (153). Proteasome-dependent degradation of CIS, most likely in the form of a (GHR-JAK2)-CIS complex, is therefore proposed to be an important step in the time-dependent CIS inhibition mechanism. The down-regulation of GHR-JAK2 signaling to STAT5b seen in GH-treated cells, as well as for cells treated with IL-3, IL-2 and EPO, could be prevented by treatment with different proteasome inhibitors (154, 155, 152), sugges-ting that SOCS proteins are key mediators of cytokine-JAK-STAT desensitization response seen in cells and tissues exposed to the different cytokines. Recently, it was demonstrated that supression of JAK2 activity was dependent on SOCS-1, where its SOCS box mediated proteasomal degradation of JAK2 rather than JAK2 kinase inhibition (156). Degradation of JAK2 depended on its phosphorylation and its high-affinity binding with SOCS-1 through the kinase inhibitory region and the SH2 domain. The SOCS box of SOCS-1 was found to interact with the E3 Cullin-2 and promoted ubiquitination of JAK2. This interaction demonstrated the substrate-specific E3 ubiquitin-ligase-like activity of SOCS-1 for activated JAK2, providing a novel strategy for the supression of oncogenic tyrosine kinases (156).

The Ubiquitin-Proteasome System

Ubiquitin conjugation: E1, E2, E3 enzymes

Ubiquitin ia a 76-amino-acid globular protein that is highly conserved throughout eukaryotes, with only three amino-acid changes from yeast to human. Ubiquitin conjugation to target proteins -ubiquitination or ubiquitylation- is essential for the degradation of proteins whose levels are regulated either constitutively or in response to changes in the cellular environment. Ubiquitin-mediated proteolysis of a variety of cellular proteins plays an important role in many basic cellular processes. Among these are regulation of cell cycle and division (157, 158, 159), diffe-rentiation and development (160, 161, 162, 163), modulation of cell-surface receptors (164, 165), involvement in the intracellular response to stress and extracellular effectors (166, 167), apoptosis (168, 169, 170), ion channels and the secretory pathway (protein transport) (171, 165), morphogenesis of neuronal networks (172), transcriptional regulation and signal transduction (173, 164, 174), transcriptional silencing, DNA repair (175, 176), long-term memory, circadian rhythms, quality control in the endoplasmic reticulum (ER) (177, 178), regulation of the immune and inflammatory responses (antigen processing) (179, 180, 181), and biogenesis of organelles (182).

Ubiquitination involves a three-step cascade mechanism. First, a ubiquitin-activating enzyme, E1, activates ubiquitin by forming a high-energy thiol-ester bond with the carboxyl-terminal glycine of ubiquitin in an ATP-dependent process. One of several E2 enzymes (ubiquitin-conjugating enzymes, UBCs) accepts ubiquitin from

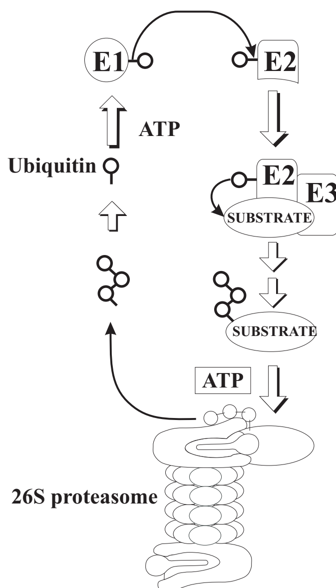

Figure 4. General model of the ubiquitin-proteasome system

the E1~S~ubiquitin intermediate by a trans-thiolation reaction, involving the carboxyl terminus of ubiquitin. An ubiquitin protein ligase, E3, catalyses the transfer of ubiquitin from the E2~S~ubiquitin intermediate to the ε-amino group of a lysine residue on the substrate. As E3 enzymes specifically bind protein substrates, they play an important role in selection of proteins for degradation. In successive reactions, a polyubiquitin chain is synthesized by processive transfer of ubiquitin moieties to Lys-48 of the previous already conjugated ubiquitin molecule (183). Polyubiquitinated proteins are then recognized and degraded by the downstream 26S proteasome complex, and free and reutilizable ubiquitin is released (reviewed in: 184, 185, 186). In many cases, however, E2 transfers activated ubiquitin directly to the protein substrate. Thus, E2 enzymes also play an important role in substrate recognition, although, in most cases, this modification is of the monoubiquitin type and is probably not involved in targeting the substrate for degradation.

E1

E1 enzyme is a homodimer with an apparent molecular mass of 210 kDa that is composed of two 105-kDa subunits (187). E1 is strongly conserved in evolution, where the human and yeast proteins are 53% identical and 73% homologous. The yeast genome encodes for a single ubiquitin-activating enzyme, UBA1, and inactivation of this gene is known to be lethal. E1 is highly concentrated in both yeast (188) and mammalian cell nuclei (189). Nuclear localization signals allow cell-cycle-regulated nuclear localization and phosphorylation, with an increase in nuclear distribution in G2 phase (190). The finding that cells expressing a tempe-

rature-sensitive E1 undergo cell-cycle arrest provided the first evidence for the physiological significance of ubiquitination (191). However, its role in the nucleus is still not clear. E1 may be involved in the ubiquitination of specific nuclear proteins such as histones or in the degradation of other nuclear proteins (192).

E2

Thirteen E2s have been isolated from yeast (termed Ubc1-13) and there are at least 25 more E2s described in other eukaryotes (175, 193). All known E2s share a 35% conservative domain of 14-16-kDa that contains the Cys residue required for the formation of ubiquitin-E2 thiol ester intermediate. Some of the E2 proteins consist almost entirely of this conserved domain, while others have C-terminal extensions that are either neutral, or highly acidic, and that promote interaction with substrates or interactions with specific E3s. Each E2 interacts with a number of E3 ligases, thus being involved in targeting numerous substrates, and the specific functions of E2s are due to their association with distinct E3s (194). Some E2s are involved in specific cellular processes while the role of others is still unclear. One group of E2 enzymes is involved in multiple ubiquitination of cellular proteins that signals them for degradation. Such enzymes are, for instance, UBC4 and UBC5, involved in the degradation of the bulk of short-lived and abnormal proteins. They are also required to enable the cell to withstand stress, such as heat shock or exposure to cadmium, probably by mediating degradation of denatured proteins generated under these conditions. A mammalian E2 homologous to yeast UBC4/UBC5 is involved in polyubiquitination and degradation of p53 and of many other proteins as well. The yeast UBC2/RAD6 is involved in degradation of "N-end rule" substrates (see below) and also in DNA repair (185). UBC3/CDC34 is required for G1 to S transition, probably via degradation of certain cell-cycle regulators. Other E2s are membrane-associated and may be involved in degradation of abnormal or virus-targeted ER proteins. Experiments in cell free systems showed that certain E2 enzymes are involved in E3-independent monoubiquitination that does not target proteins for degradation. However, their physiological significance is not clear (194).

E3

E3s bind the target substrates, either directly or indirectly, via ancillary proteins, and catalyze 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 it. Until now, only a few E3s were actually discovered. Despite the importance of E3s in determining the specificity of the system, there is no sequence homology among different E3s, and the frequent association of these enzymes with multisubunit complexes in which the identity of the ligase subunit is not known, makes these proteins difficult to study.

Until now, three families of E3s were described:

(i) The main N-end rule E3, E3α, and its yeast counterpart UBR1, contain two distinct sites that recognize either basic (Type I) or bulky-hydrophobic (Type II) N-terminal residues of their substrates. However, they also recognize non-N-end rule substrates such as N-α-acetylated proteins that bind via another site (186, 195).
(ii) The second group of E3s, HECT E3s, were first discovered when oncogenic strains of human papillomavirus (HPV) were encoding isoforms of a protein called E6, which specifically inactivated the tumor suppressor protein p53. E6 functions as an adaptor between E6-associated protein (E6-AP), a cellular partner for E6, and p53, allowing E6-AP to ubiquitinate p53 (196). E6-AP and a family of proteins closely related to E6-AP in a 350-residue region at their carboxyl termini, termed the HECT domain, were further identified (197). This domain includes a conserved cysteine that forms a covalent thiol-ester intermediate with ubiquitin (197). With the exception of E6-AP, the HECT family of E3s contains N-terminal WW domains. These domains are known to be involved in protein-protein interactions, via proline-rich motifs of proteins, and in targeting substrates for ubiquitination (198). Most of these WW domain HECT3s include an N-terminal C2 domain, known to mediate translocation to the plasma membrane in response to increases in intracellular Ca^{2+}, like for instance Nedd4 (199). Nedd4 is one rare example of an E3 enzyme ubiquitinating a membrane protein, the kidney epithelial sodium channel (ENaC) (200). Nedd4 binds directly through its WW domain to ENaC and induces its ubiquitination and degradation in the lysosome, leading to downregulation of the number of active channels. Mutation of proline-rich regions of ENaC, known to interact with Nedd4, causes Liddle syndrome, an inherited form of hypertension in which ENaC activity is increased due to the inability of Nedd4 to downregulate ENaC (198, 201, 202).
(iii) A third type of ligase are the RING finger-containing E3s (203, 204). RING fingers have been defined by a pattern of conserved Cys and His residues that form a cross-brace structure that probably binds two zinc cations (205). This group of proteins is rather large, with molecules forming single and multisubunit E3s. The single-subunit E3s contain both a RING finger domain and the substrate binding and recognition site on the same molecule. Examples of these proteins include the oncoprotein Mdm2, responsible of ubiquitinating p53 (206, 207), the proto-oncoprotein c-Cbl, responsible for ubiquitinating growth factor receptors (208, 209, 210), and the inhibitors of apoptosis (IAPs) (211). Multisubunit E3s consist of a small RING finger protein and a member of the cullin family of proteins as well as other subunits, some of which recognize substrates. Among this E3 group are the anaphase promoting complex (APC)/cyclosome which includes at least 12 distinct subunits and is involved in degradation of cell cycle regulators (212, 213, 214); the Skp1-Cullin/Cdc53-F-box protein (SCF)-RING finger complexes involved in degradation of signal- and cell cycle-induced phosphorylated proteins (215); and the von-Hippel Lindau (pVHL)-Elongins B and C (VBC)-Cul2-RING finger complex (216, 217,

218) that targets hypoxia-inducible transcription factor 1α (HIF-α) and possibly JAKs for degradation (219, 151), see above: SOCS proteins). The APC complex has an ubiquitin ligase activity specific for cell-cycle regulators, like for instance mitotic cyclins, some anaphase inhibitors and spindle-associated proteins, that contain a nine amino acid motif termed the "destruction box" and are degraded during mitosis. Normally, the complex is only activated at the end of mitosis by phosphorylation that is mediated by the cyclin-B/cyclin-dependent kinase (CDK)1 complex M-phase promoting factor (MPF) (220). The SCF E3s recognize and ubiquitinate several phosphoproteins, with the key substrate recognition element being dependent on the F-box protein component of the complex (221).
The SCF complexes act along with the E2 Cdc34/Ubc3, with the following structure: E2-Rbx1/Roc1-Cdc53/Cullin-1-Skp1-F-box protein-protein substrate. There are examples in which one F-box protein is able to recognize different substrates, as in the case for β-transducin-repeat-containing protein (βTRCP) that recognizes both β-catenin (222), IkBα (223), and HIV-1-Vpu (224). In spite of the fact that the plasma membrane Vpu undergoes phosphorylation and recruits E3, it is the HIV receptor CD4 that is targeted for degradation in the ER membrane following formation of a CD4-Vpu-SCF complex. Furthermore, some F-box proteins are themselves ubiquitinated and degraded, suggesting a mechanism of regulating SCF function and facilitating proteasomal targeting of associated target proteins. In contrast, the ubiquitination of the transcription factor Met4 by SCFMet30 serves to regulate its activity but not its proteolysis (225). The VBC complex is quite comparable to the SCF complex, containing Elongins B and C, Cullin 2, and pVHL, in addition to RBX1/Roc1, the same RING finger protein that is shared by SCF complexes (219). Skp1 is replaced by the dimer of elongin B, which has homology with Skp1, and elongin C, which is an UDP. The substrate recognizing subunit has not yet been identified, being pVHL the best candidate. Similar to the SCF complex, it is possible that more than one substrate recognition proteins are involved in BC-Cul2-Rbx1 E3 complexes. Analogous to the F box, the VHL protein contains a SOCS box that interacts with the core of this E3. It might be that other SOCS-containing proteins can replace the VHL and allow for recognition of other specific substrates (134, see above: SOCS proteins).

E4, U-box containing proteins, and deubiquitinating enzymes (DUBs)

In yeast, it was found that an ubiquitin chain elongation factor, E4, binds to ubiquitin moieties of pre-formed short conjugates and catalyses ubiquitin chain elongation in conjunction with E1, E2, and E3 (226). This new family of proteins shares a modified version of RING finger designated U box that lacks the metal-chelating residues typical of RING fingers (227). The function of the U box is, however, still not clear. Possibly, it might recruit E2 to the conjugation machinery or bind to the short ubiquitin chain attached to the substrate protein. One example is the CHIP

protein, known to be involved in both the degradation of the cystic fibrosis transmembrane conductance regulator (CFTR), which is a chloride ion channel, and the glucocorticoid receptor together with the co-chaperones Hsc70 and Hsp90, respectively (228, 229).
Covalent attachment of ubiquitin to proteins is a reversible process, through the action of deubiquitinating enzymes (DUBs). There are 19 yeast DUBs and several more in mammals discovered until now. These enzymes are cystein proteases that specifically hydrolyze the amide bond immediately after the C-terminal residue. DUBs belong to a large and diverse family of enzymes divided as UHCs (ubiquitin carboxyl-terminal hydrolases) and UBPs (ubiquitin-specific processing enzymes) (230, 231). UHCs are 20-30 kDa enzymes that remove short or flexible peptide chains from the C-terminus of ubiquitin. UBPs, on the other hand, are enzymes of about 100 kDa, thought to remove ubiquitin ubiquitin form larger proteins, and are involved in the disassembly of multi-ubiquitin chains. Both types of DUBs play several roles in maintaining the steady-state levels of free ubiquitin and in affecting the stability of ubiquitin-conjugated proteins (232, 230, 231). For instance, generation of ubiquitin, recycling of ubiquitin, maintaining proper levels of ubiquitin in the cell, editing polyubiquitin chains, and helping in proteasome-dependent degradation and proteasome-dependent endocytosis, are processes regulated by DUBs (233, 234).

The Proteasome

The proteasome is a 700,000-dalton (~2.5 MDa), cylinder-shaped multimeric dimer protease arranged as four axially stacked heptameric rings (235). Its proteolytic active sites are situated in a cylindrical chamber, the 20S core, which has a 19S regulator complex, also called PA700 or cap complexes, at either end (236). The complex of one 20S and two 19S subunits form the 26S proteasome. The 20S core particle contains seven evolutionarily related, but not identical, subunits, that include two homologous gene products, α and β (237). The two outer rings are composed of α-subunits (α1-α7), which form the "gates" through which substrates enter and products are released, whereas the two inner rings are composed of β-subunits (β1-β7) (238). Three of these β-subunits, β1, β2, and β5, contain the active sites. The catalytically active residue is a single threonine located at the amino termini of the three β-subunits (239), and characterizes the proteasome as a member of the family of amino-terminal nucleophile hydrolases (240, 241). Each of the three β-subunits has a different preference for cleavage, characterizing the proteasome with three activities with distinct specificities against short synthetic peptides: a "chemotryptic-like" activity with preference for tyrosine or phenylalanine at the P1 position; a "tryptic-like" activity with preference for arginine or lysine at the P1 position; and a "postglutamyl" hydrolyzing activity with a preference for glutamate or other acidic residues at the P1 position (242, 243).

The 19S regulatory particle plays several roles in regulating proteasomal activity, including substrate selection, translocation into the core particle, as well as influencing the nature of products generated by the core particle. The 19S regulator has two multisubunit components, one constitutes the base and the other the lid. The base, composed of six ATPases of the AAA family plus two non-ATPases subunits (244), binds to the 20S core. The ATPases function as chaperones to help unfolding substrates and to transfer them into the 20S core, thereby controlling access of substrates to the proteases within (245, 246, 247). The lid, on its turn, is composed of eight different non-ATPase subunits with unknown function.
The proteasome recognizes its substrates via the ubiquitin chains (248). The proteasome degrades proteins into small peptides of about seven to nine amino acids, which can, in turn, be hydrolyzed to amino acids by cytosolic peptidases. The 26S proteasome is responsible for the degradation of abnormal and damaged proteins (243), of oncogenes and tumor suppressors (249), of cell-cycle regulators (250, 251), in the processing of antigens (252), and in the activation or degradation of transcription factors (253, 254). The essential function of the proteasome in these processes was first discovered by the analysis of inactive mutants and the application of specific inhibitors (255, 180, 256). Covalent attachment of lactacystin, a *Streptomyces cerevisiae* metabolite, to the catalytically active threonine of β-subunits irreversibly inhibits proteolytic activity, indicating that this residue is essential for catalysis and that lactacystin or, in fact, clasto-lactacystin β-lactone, a product of lactacystin hydrolysis that actually reacts with the proteasome (257), can be used as a powerful tool for proteasome inhibition (255). Other covalent inhibitors are peptidyl vinyl sulfones (258), or dipeptidyl boronic acids (259). Reversible inhibitors of the proteasome include peptide aldehydes (260). Most of the proteasome inhibitors act by reacting with the threonine of the β subunits (249).
The 26S proteasome was shown to degrade misfolded or damaged secretory proteins from the ER in an ubiquitin- and ATP-dependent way in the cytosol, a mechanism known as the ER-associated degradation pathway (ERAD) (261, 262). By use of specific localization signals, the 26S proteasome can be transported to different locations in the cytosol or nucleus, depending where his action is needed (263, 264). Besides completely degrading most of polyubiquitinated proteins, in some cases only partial degradation occurs. In case of the inducible transcription nuclear factor κB (NF-κB), responsible for regulating the transcription of several cellular genes involved in the immediate early processes of acute phase, inflammatory and immune responses, the proteasome partially degrades the ubiquitinated C-terminal domain form of the p105 precursor protein (265, 266). Partial processing of p105 leads to the formation of the p50 protein, which will then interact with RelA (p65) to form an active NF-κB, which on its turn, will translocate to the nucleus to induce its activity. Partial degradation of p105 suggests that certain amino acid sequence in this protein, a glycine-rich region, can induce its release from the proteasome (267).

Ubiquitination of Membrane Proteins

ER membrane proteins

As mentioned above, ERAD serves an important role in cellular defense and quality control by targeting abnormal/misfolded proteins, or excess of un-assembled subunits of multi-subunit complexes, for ubiquitination and proteasome degradation. These proteins undergo a retrograde transport from the ER to the cytosol, also termed dislocation, by using the same translocon in opposite direction (268, 269). The protein channel mediating these transports is the translocon, composed of the trimeric ER membrane protein Sec61, the heterodimeric signal-sequence recognition particle (SRP) receptor, and in some cases the translocating chain-associating membrane (TRAM) protein (270). Several ER proteins have been shown to require specific E2s and to become ubiquitinated for degradation by the proteasome, like for instance ENaC (200), mutant sec61 (271, 272), Na.K-ATPase α subunits (273), and apolipoprotein B (274). Among other membrane proteins degraded via ERAD are the yeast and human 3-hydroxyl-3-methyl-coenzyme A reductase (HMGR) (275, 276), the mutant prion protein (277), the Wilson disease Cu^{2+} transporter protein (278), normal and mutated CFTR (279), and MHC class I molecules targeted for degradation by the cytomegalovirus-encoded proteins US2 and US11 (280).

Plasma membrane proteins

Several permeases and transporters, like ENaC and yeast Gap1p, and cell-surface receptors, are internalized and degraded in a ligand-dependent and ubiquitin-dependent manner (164, 281, 282, 200, 201, 283, 284). In most cases, the proteasome does not recognize these ubiquitinated proteins, suggesting that ubiquitination functions in downregulating such proteins from the cell-surface. Ubiquitination appears to activate the internalization and endocytic machineries that route the internalized proteins to the vacuole/lysosome, where they are degraded. Possibly, ligand binding to its receptor triggers either exposure of the ligase binding site(s) or phosphorylation sites. In the latter case, phosphorylation then recruits the conjugation machinery. The mechanisms by which ubiquitin triggers internalization of membrane proteins are still unknown. Normally, internalization is triggered by tyrosine- or di-leucine motifs, which interact with the clathrin adaptor protein AP-2 (285, 286), resulting in clathrin activation and coated pit formation. These clathrin-coated buds will then pinch off from the plasma membrane and form clathrin-coated vesicles. Analogous to AP-2, ubiquitin could act as an adaptor between lysine residues in membrane proteins and clathrin, or between amino acid sequences in the ubiquitin molecule itself and AP-2 molecules.
In yeast, G protein-coupled α-factor receptors, like Ste2p (281) and Ste3p (287), and transporters, like uracil permease Fur4 (288, 289), the maltose permease Mal61

(290), the galactose transporter Gal2p (291), and the ABC peptide transporter Ste6p (292), are ubiquitinated upon ligand binding. Ubiquitination of these proteins marks them for proteolysis, resulting in their internalization and degradation in the vacuole, the yeast equivalent of the lysosome.

Monoubiquitination of certain proteins, like Ste2p and Gal2p, has been shown to be sufficient to trigger endocytosis, suggesting that ubiquitination of such proteins is not involved in proteasomal degradation, since monoubiquitinated proteins are not a target for the proteasome (183). Fur4p seems to be modified by polyubiquitin chains, but the ubiquitin moieties are linked via Lys63, not Lys48 that is commonly used for ubiquitin attachment (a linkage that appears to be necessary for maximal rates of endocytosis) (288). Since endocytosis of Fur4p requires the "destruction box", it seems likely that ubiquitination motifs necessary for the degradation of certain proteins are also involved in endocytosis of other proteins (284).

In mammalian cells, it was shown for the Met receptor (293) and the platelet-derived growth factor (PDGF) receptor (294, 295) that ubiquitination might initiate proteasome action. Ubiquitinated forms of these proteins were observed in endosomal/lysosomal compartments (296, 297), suggesting that the proteasome is involved in a partial degradation of the cytosolic tails, while the lumenal and transmembrane domains are degraded in the lysosome. Until now, the role of the ubiquitin system in endocytosis and degradation of the receptors for immunoglobulin E (IgE) (298), CSF-1 (294), SLF (299) and the T cell antigen receptor (300), is unclear. However, ubiquitination of ENaC and GHR is clearly involved in their endocytosis, where ubiquitination acts as an internalization signal. Internalization of ENaC depends on ubiquitination of its α- and γ-subunits by the HECT domain E3 Nedd4 (200,301), and its rapid degradation is prevented by inhibitors of both the proteasome and lysosome (200). It was suggested that the assembled ENaC complex subunits might be degraded by the lysosome, while unassembled subunits could be degraded by the proteasome. For the GHR, it was shown that ligand-dependent GHR endocytosis depends on an intact ubiquitin system (83, 302).

Several steps in the internalization/degradation pathway could be regulated by ubiquitination. In some cases, it is not the receptor, but another nonreceptor protein that requires ubiquitination. For instance, Eps15, a protein that becomes receptor-associated, phosphorylated, ubiquitinated, and localized to the clathrin-coated pits upon ligand binding of the EGFR (303, 304, 305). In other cases, ubiquitination plays a role in the endosomal sorting pathway. Some receptor ligands such as EGF, PDGF and colony stimulating factor (CSF-1), induce the ubiquitination both of their receptors and of a tyrosine kinase regulator, c-cbl (306, 307, 308). C-cbl has been shown to participate in the endosomal sorting by binding and enhancing the ubiquitination of the cytoplasmic domain of the endocytosed receptors (309). Such ubiquitinated receptors are localized to the lysosome for degradation, while those that are not ubiquitinated are recycled to the cell-surface. The precise role of the proteasome in

this membrane sorting process is still unclear. Involvement of proteasomes in the degradation of cytosolic tails has not been established for any of the described proteins. Degradation products in the absence or presence of specific proteasome inhibitors have not been described, not even when antibodies were directed against the extracellular 'proteasome-undegradable' part of these cell-surface proteins. This does not exclude proteasome action, as the proteasome might degrade the cytosolic domains of these proteins gradually, during their transport from the plasma membrane to the lysosome. This would result in degradation intermediates of different lengths, which are not easily detectable by immunoblotting (284).

Ubiquitin system-dependent endocytosis of the GHR

In the presence of ligand GHR endocytoses rapidly via clathrin-coated pits (310), and its degradation occurs at least partially within the lysosome (311). The GHR was initially found ubiquitinated upon amino acid sequencing of the receptor from rabbit liver (11). The ubiquitin system is required for ligand-induced GHR internalization (83). In a chinese hamster cell line carrying a temperature-sensitive E1 enzyme (ts20 cells), inactivation of E1 results in accumulation of non-ubiquitinated GHRs at the plasma membrane, while internalization of the transferrin receptor is unaffected (83). In contrast to several other proteins (200, 312), ubiquitination itself is not important for endocytosis, as substitution of all the Lys residues in the cytosolic tail did not affect the process (313). Yet, the ubiquitin conjugation machinery is still essential for endocytosis, and a 10 amino acid sequence in the tail, designated ubiquitin endocytosis (UbE) motif and involved in both GHR ubiquitination and ligand-induced endocytosis (313), is required for this activity. Probably, this motif serves, directly or via adaptor proteins, as an anchoring site for possible E2s/E3s, leading to coated pit localization and subsequently to GHR internalization. Upon binding of the ubiquitinating enzymes, interaction with an endocytic adaptor, e.g. AP2 could occur, or alternatively, the E2/E3 complex could serve as an adaptor, analogous to the role of arrestin for the β-adrenergic receptor (314). If ubiquitination of a GHR-associated protein is involved in the endocytosis process, possible candidates could be Eps15 and c-Cbl, as mentioned above.The UbE motif does not resemble any other known ubiquitination domain (313), and mutation of the aromatic as well as the acidic residues did not allow internalization. In particular, the phenylalanine at position 327 mutated to an alanine blocked internalization. Possible candidates containing an UbE motif and reported to be also ubiquitinated are for instance, the prolactin receptor (315), the PDGF receptor (316), and the c-erbB-2 receptor (317).
Recently, the proteasome has been shown to also be involved in GHR downregulation (318). GHR internalization requires proteasomal action in addition to an active ubiquitin conjugation system. Specific proteasomal inhibitors block GH uptake of the full-length GHR, while a truncated receptor can endocytose undisturbed (318).

Furthermore, if the GHR is truncated at position 349, a latent di-leucine endocytosis motif becomes active and the UbE motif is no longer required for ligand-induced internalization (313). This suggests that upon removal of a portion of the tail, this di-leucine motif is activated, and therefore, the GHR cytosolic tail is cut from the C-terminus.

The ubiquitin system and the stress response

Stresses such as elevated temperatures, heavy metals, amino acid analogs, viral infection, oxidative and chemical damage, or the production of abnormal proteins trigger vital cellular responses, either to repair the damage, or to limit its toxicity to the cell. Normally, transcriptional induction of a set of genes whose products, known as stress proteins, enhances survival under stress conditions (for review: 319). The synthesis of heat shock proteins at sub-heat shock temperatures is triggered by the forced production of denatured proteins (320), indicating that protein damage is the key event in the induction of the stress response. When the ubiquitin pathway was described as a mechanism for selective degradation of abnormal proteins containing amino acid analogs (321, 322, 191), it first confirmed its important role in stress response. Subsequently, certain components of the pathway were found to be stress proteins, and ubiquitin conjugation was increased in heat shock and other stress conditions. Ubiquitin itself is a stress protein, where its expression is induced by diverse stresses, including heat shock (323), heavy metals (arsenium and cadmium) (178), amino acid analogs (323), DNA-damaging agents (324) and oxidants (325). Imposition of stress is usually accompanied by a dramatic redistribution of ubiquitin, such that the fraction of ubiquitin that is unconjugated decreases, while the fraction of ubiquitin in conjugates increases (323, 327, 328). The requirement of ubiquitin expression in stressed cells reflects the need to replenish the pool of free ubiquitin, and in the absence of such replenishment, the ubiquitination of denatured proteins is inhibited and these species accumulate to toxic levels. Expression of specific E2 enzymes is also up-regulated in stressed cells (326). In yeast, UBC5 is important for the ubiquitination and turnover of normal-short-lived proteins, but also in heat shock and cadmium induced stress (326, 178). No E3 enzymes have yet been identified as stress proteins, nor has any E3 been shown to play an important role in the stress response. The effect of stress can be extreme: in unstressed cells, about 50% of the ubiquitin is unconjugated (329), whereas less than 10% of the ubiquitin is unconjugated in heat-shocked HeLa cells (330). The use of protein synthesis inhibitors like cycloheximide, does not prevent stress-induced ubiquitin redistribution, suggesting that this event is mainly due to increased susceptibility of proteins to ubiquitination. However, up-regulation of ubiquitin and E2s might also contribute to increased conjugate levels (328).
Upon oxidative stress, both protein ubiquitination and proteasomal degradation have been demonstrated (328, 331). Oxidative stress damages cells, and has been

implicated in several processes, including degenerative diseases of aging, Alzheimer's and Parkinson's disease, arthritis, atherosclerosis, and cancer (332, 333, 334, 335, 336, 337). The exposure of cells to high levels of oxygen free radicals results in damage of proteins, lipids, DNA, and enzyme activity, affecting both cell proliferation and differentiation. Proteins containing tiol groups/Cys residues are sensitive targets of oxidants, where, for instance, phosphorylation /dephosphorylation activities are affected. Several proteins involved in signal transduction, including MAPKs, Raf-1, Ras, and growth factors receptors, such as EGFR and PDGFR, are phosphorylated and activated upon oxidative stress (338, 339, 340, 341, 342). It is thought that the increased EGFR phosphorylation upon hydrogen peroxide (H_2O_2) exposure, is a result of tyrosine phosphatase inactivation (341, 343). Following cellular exposure to oxidants, the levels of the primary cellular sulfhydryl reductant glutathione (GSH) decrease, while the levels of its oxidized form (GSSG) increase. Upon severe oxidative stress, ubiquitination of proteins is also decreased, suggesting that oxidative stress interferes with E1/E2 activities (344, 345). These enzymes contain active site sulfhydryls that might be covalently modified (thiolated) upon enhancement of GSSG levels (glutathiolation). Glutathiolation might regulate the levels of ubiquitinated proteins in response to oxidative stress (345). Furthermore, the 20S proteasome was found more resistant to oxidative stress than the ATP- and ubiquitin-dependent 26S proteasome (346).
Ubiquitin-dependent receptor endocytosis is also affected by oxidative stress. Both internalization of N-acetlyglucosamine-specific chicken hepatic lectin (CHL) receptor (347), and EGFR (348) were shown to become inhibited upon H_2O_2 stress. Possibly, H_2O_2 inhibits EGFR internalization by inhibition of Eps15 ubiquitination, known to be involved in the internalization process (348). Whether GHR constitutive levels at the plasma membrane are also affected by stress-induced ubiquitination, is a question to be answered.

Scope of this thesis

The ubiquitin-proteasome system is known to be involved in GHR endocytosis, where an active ubiquitin system is necessary for receptor ubiquitination and its consequent internalization. Endocytosis of the GHR has also been shown to be regulated by the proteasome. This thesis focuses, first of all, on the effect of the ubiquitin system on the GHR signal transduction and downregulation. In chapter 2, we studied the role of the ubiquitin system in the signal transduction of the GHR. By using specific proteasome inhibitors, we determined their effect on GH-induced activity of both GHR and JAK2 in both the wild-type GHR and in the internalization-deficient GHR mutant F327A. We suggest possible models for the role of the ubiquitin-proteasome system in GHR signal transduction. Furthermore, we asked the question involving the continuation of signal transduction of the GHR after its

internalization, where binding of JAK2 as well as other activated proteins to the GHR, not only at the cell-surface but also intracellularly, was determined. Degradation products of the GHR were determined in both cell-surface and in endosomes, indicating that part of the receptor is down-regulated by the proteasome.
In chapter 3, we focused on the role of JAK2-mediated signal transduction in GHR internalization and downregulation. Using a box-1 mutant of the GHR, activation of both GHR and JAK2 was determined, as well as receptor-bound GH internalization and GHR turnover and transport to the plasma membrane. We determined the role of GHR signal transduction, via JAK2, on GHR ubiquitination, endocytosis and degradation, and the role of the ubiquitin system on the regulation of both GHR internalization and signal transduction.
Finally, chapter 4 discusses the role of stress in regulating GHR levels in the cell. We determined the effect of H_2O_2 on receptor's turnover and on receptor's down-regulation from the cell-surface. We further determined the effect of oxidative stress on the amount of ligand binding sites at the cell-surface and related these results with the enhancement of shedding activity directed to the GHR.

References

1. Bazan, J. F. (1989) Biochem. Biophys. Res. Commun. 164, 788-795
2. Finidori, J., and Kelly, P. A. (1995) J. Endocrinol. 147, 11-23
3. Cosman, D., et al. (1990) Trends Biochem. Sci 15, 265-270
4. Kelly, P. A., Djiane, J., Postel-Vinay, M. C., and Edery, M. (1991) Endocr. Rev. 12, 235-251
5. Miyazaki, T.,.et al. (1991) EMBO J. 10, 3191-3197
6. Duriez, B., et al. (1993) Mol. Endocrinol. 7, 806-814
7. De Vos, A. M., Ultsh, M., and Kossiakoff, A. A. (1992) Science 255, 306-312
8. Murakami, M., et al. (1991) Proc. Natl. Acad. Sci. USA 88, 11349-11353
9. Tanner, J. W., et al. (1995) J. Biol. Chem. 270, 6553-6530
10. Argetsinger, L. S., et al (1993) Cell 74, 237-244
11. Leung, D. W., et al. (1987) Nature Lond. 330, 537-543
12. Fuh, G., et al. (1990) J. Biol. Chem. 265, 3111-3115
13. Gobius, K. S., et al. (1992) J. Mol. Endocrinol. 9, 213-220
14. Wells, J. A., et al. (1993) Rec. Prog. Horm. Res. 48, 253-275
15. Harding, P. A., et al. (1994) Mol. Cell. Endocrinol. 106, 171-180
16. Dastot, F., et al. (1996) Proc. Natl. Acad. Sci. USA 93, 10723-10728
17. Ross, R. J. M., et al. (1997) Mol. Endocrinol. 11, 265-273
18. Smith, W. C., Kuniyoshi, J., and Talamantes, F. (1989) Mol. Endocrinol. 3, 984-990
19. Edens, A., Southard, J. N., and Talamantes, F. (1994) Endocrinology 135, 2802-2805
20. Zhang, Y., et al. (2000) Endocrinology 141, 4342-4348
21. Baumann, G., et al. (1987) J. Clin. Endocrinol. Metab. 64, 657-660
22. Clark, R. G., et al. (1996) Endocrinology 137, 4308-4315
23. Mannor, D. A., et al. (1991) J. Clin. Endocrinol. Metab. 73, 30-34
24. Baumann, G. (1994) J. Endocrinol. 141, 1-6
25. Tannenbaum, G. S. (1991) Acta Paediatr. Scand. Suppl. 372, 5-16
26. Isaksson, O. G., Eden, S., and Jansson, J. O. (1985) Annu. Rev. Physiol. 47, 483-499

27. Casanueva, F. F. (1992) Endocrinol. Metab. Clin. North. Am. 21, 483-517
28. Davidson, M. B. (1987) Endocr. Rev. 8, 115-131
29. Evock, C. M., Etherton, T. D., Chung, C. S., and Ivy, R. E. (1988) J. Anim. Sci. (66), 1928-1941
30. Rudman, D., et al. (1988) N. Engl. J. Med. 323, 1-6
31. Corpas, E., Harman, S. M., and Blackman, M. R. (1993) Endocr. Rev. 14, 20-39
32. Chen, H. T., Schuler, L. A., and Schultz, R. D. (1998) Mol. Cell. Endocrinol. 137, 21-29
33. Yoshizato, H., et al. (1998) Endocrinol. 139, 2545-2551
34. Lombardi, G., et al. (1997) J. Pedriatr. Endocrinol. Metab. 10, 553-560
35. Calao, A., et al. (1997) J. Clin. Endocrinol. Metab. 82, 2777-2781
36. Dietz, J., and Schwartz, J. (1991) Metabolism 40, 800-806
37. Lewis, U. J., Sinha, Y. N., and Haro, L. S. (1994) Acta Pediatrica 399, 29-31
38. Goffin, V., Shiverick, K. T., Kelly, P. A., and Martial, J. A. (1996) Endocrinol. Rev. 17, 385-410
39. Chung, C. S., and Etherton, T. D. (1986) Endocrinol. 119, 780-786
40. Gluckman, P. D., Breier, B. H., and Sauerwein, H. (1990) Acta Paediatr. Scand. Suppl. 366, 73-78
41. Posner, B. I., Patel, B., Vezinhet, A., and Charrier, J. (1980) Endocrinol. 107, 1954-1958
42. Lesniak, M. A., and Roth, J. (1976) J. Biol. Chem. 251, 3720-3729
43. Nilsson, A., et al. (1990) Mol. Cell. Endocrinol. 70, 237-246
44. Bornfeldt, K. E., et al. (1989) J. Endocrinol. 122, 651-665
45. Straus, D. S., and Takemoto, C. D. (1990) Mol. Endocrinol. 4, 91-100
46. Brameld, J. M., Gilmour, R. S., and Buttery, P. J. (1999) J. Nutr. 129, 1298-1306
47. Waters, M. J., et al. (1994) Proc. Soc. Exp. Biol. Med. 206, 216-220
48. Fuh, G., et al. (1992) Science 256, 1677-1680
49. Harding, P. A., et al. (1996) J. Biol. Chem. 271, 6708-6712
50. Clackson, T., and Wells, J. A. (1995) Science 267, 383-386
51. Argetsinger, L. S., and Carter-Su, C. (1996) Physiol. Rev. 76, 1089-1107
52. Thirone, A. C., Carvalho, C. R., and Saad, M. J. (1999) Endocrinol. 140, 55-62
53. Smit, L. S., et al. (1996) Mol. Endocrinol. 10, 519-533
54. Jiang, et al. (1998) Biochem. Biophys. Res. Commun. 253, 774-779
55. Harpur, A. G., et al. (1992) Oncogene 7, 1347-1353
56. Frank, S. J., Yi, W., Zhao, Y., et al. (1995) J. Biol. Chem. 270, 14776-144785
57. Colosi, P., Wong, K., Leong, S. R., and Wood, W. I. (1993) J. Biol. Chem. 268, 12617-12623
58. Sotiropoulos, A., et al. (1994) Endocrinology 135, 1292-1298
59. VanderKuur, J. A., et al. (1994) J. Biol. Chem. 269, 21709-21717
60. Dinerstein-Cali, H., et al. (1995) Mol. Endocrinol. 9, 1701-1707
61. Wang, X., et al. (1996) Mol. Endocrinol. 10, 1249-1260
62. Silvennoinen, O., et al. (1993) Sicence Wash. DC 261, 1736-1739
63. Carter-Su, C., and Smit, L. S. (1998) Recent Prog. Horm. Res. 53, 61-83
64. Rui, L., et al. (1997) Mol. Cell Biol. 17, 6633-6644
65. Billestrup, N., et al. (1995) Proc. Natl. Acad. Sci. USA 92, 2725-2729
66. Moldrup, A., et al. (1991) J. Biol. Chem. 266, 17441-17445
67. Goujon, L., et al. (1994) Proc. Natl. acad. Sci. USA 91, 957-961
68. Xu, B. C., Wang, X., Darius, C. J., and Kopchick, J. J. (1996) J. Biol. Chem. 271, 19768-19780
69. Lobie, P. E., et al. (1995) J. Biol. Chem. 270, 21745-21750
70. Darnell, J. E. J., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415-1421
71. Schindler, C., and Darnell, J., J. E. (1995) Annu. Rev. Biochem. 64, 621-651
72. Finidori, J. (2000) Vitamins and Hormones 59, 71-97
73. Darnell, J. E., Jr. (1997) Science 277, 1630-1635
74. Shuai, K., et al. (1993) Science 261, 1744-1746
75. Zhong, Z., Wen, Z., and Darnell, J. E. (1994) Science Wash. DC 264, 95-98

76. Akira, S., et al. (1994) Cell 77, 63-71
77. Wegenka, U. M.,et al. (1994) Mol. Cell. Biol. 14, 3186-3196
78. Wakao, H., Gouilleux, F., and Groner, B. (1994) EMBO J. 13, 2182-2191
79. Ihle, J. N. (1996) Cell 84, 331-334
80. Gronowski, A. M., and Rotwein, P. (1994) J. Biol. Chem. 269, 7874-7878
81. Campbell, G. S., et al. (1995) J. Biol. Chem. 270, 3974-3979
82. Wood, T. J., Sliva, D., Lobie, P. E., et al. (1995) J. Biol. Chem. 270, 9448-9453
83. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. 15, 3806-3812
84. Waxman, D. J., Ram, P. A., Park, S. H., and Choi, H. K. (1995) J. Biol. Chem. 270, 13262-13270
85. Dinerstein-Cali, H., et al. (2000) Mol. Cell. Endocrinol. 166, 89-99
86. Sotiropoulos, A., et al. (1995) FEBS Letters 369, 169-172
87. VanderKuur, J., et al. (1995) J. Biol. Chem. 270, 7587-7593
88. Hansen, J. A., et al. (1997) J. Mol. Endocrinol. 18, 213-221
89. Anderson, N. G. (1992) Biochem. J. 284, 649-652
90. Campbell, G. S., Miyasaka, T., Pang, L., and Saltiel, A. R. (1992) J. Biol. Chem. 267, 6074-6080
91. Winston, L. A., and Bertics, P. J. (1992) Mol. Cell Biol. 11, 2057-2065
92. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843-14846
93. Winston, L. A., and Hunter, T. (1995) J. Biol. Chem. 270, 30837-30840
94. Vanderkuur, J. A., et al. (1997) Endocrinol. 138, 4301-4307
95. Yamauchi, T., et al. (1997) Nature 390, 91-96
96. Souza, S. C., et al. (1994) J. Biol. chem. 269, 30085-30088
97. Argetsinger, L. S., et al. (1995) J. Biol. Chem. 270, 14685-14692
98. Sun, X. J., et al. (1995) Nature Lond. 377, 173-177
99. Kuhne, M. R., et al. (1993) J. Biol. Chem. 268, 11479-11481
100. Lee, C.-H., et al. (1993) Proc. Natl. Acad. Sci. USA 90, 11713-11717
101. Myers, M. G., et al. (1994) Mol. Cell. Biol. 14, 3577-3587
102. Skolnik, E. Y., et al. (1993) EMBO J. 12, 1929-1936
103. Cheatham, B., et al. (1994) Mol. Cell Biol. 14, 4902-4911
104. Songyang, Z., et al. (1997) Proc. Natl. Acad. Sci. USA 94, 11345-11350
105. Moutoussamy, S., et al. (1998) J. Biol. Chem. 273, 15906-15912
106. Riddersrale, M., and Tornqvist, H. (1994) Biochem. Biophys. Res. Commun. 203, 306-310
107. Smal, J., and De, M. P. (1987) Biochem. Biophys. Res. Commun. 147, 1232-1240
108. Tollet, P., et al. (1991) Mol. Endocrinol. 5, 1315-1358
109. Gaur, S., Yamagushi, H., and Goodman, H. M. (1996) Am. J. Physiol. 270, C1485-C1484
110. Clarkson, R. W. E., et al. (1995) Mol. endocrinol. 9, 108-120
111. Dekker, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73-77
112. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329-343
113. Doglio, A., et al. (1989) Proc. Natl. Acad. Sci. USA 86, 1148-1152
114. Rogers, S. A., and Hammerman, M. R. (1989) Proc. Natl. Acad. Sci. 86, 6363-6366
115. Catalioto, R. M., et al. (1990) Biochem. Biophys. res. Commun. 173, 840-848
116.Toker, A., et al. (1994) J. Biol. Chem. 269, 32358-32367
117. Okada, S., and Kopchick, J. J. (1995) Proc. Annu. meet. Endocr. Soc. 77th Washington DC, 80
118. Roupas, P., et al. (1995) Proc. Annu. meet. Endocr. Soc. 77th Washington DC, 346
119. Schwartz, Y., Yamagushi, H., and Goodman, H. M. (1992) Endocrinol. 131, 772-778
120. Gebert, C. A., Park, S. H., and Waxman, D. J. (1999) Mol. Endocrinol. 13, 38-56
121. Sotiropoulos, A., et al. (1996) Mol. Endocrinol. 10, 998-1009
122. Hackett, R. H., et al. (1997) J. Biol. Chem. 272, 111280-11132
123. Ram, P. A., and Waxman, D. J. (1997) J. Biol. Chem. 272, 17694-17702

124. Kim, S.-O., Jiang, J., Yi, W., Feng, G. S., and Frank, S. J. (1998) J. Biol. Chem. 273, 2344-2354
125. Yin, T., Shen, R., Feng, G. S., and Yang, Y. C. (1997) J. Biol. Chem. 272, 1032-1037
126. Kishihara, K., et al. (1993) Cell 74, 143-156
127. Irie-Sasaki, J. S., T., et al. (2001) Nature 409, 349-354
128. Fernandez, L., et al. (1998) Endocrinol. 139, 1815-1824
129. Aman, M. J., and Leonard, W. J. (1997) Curr. Biol. 7, R784-R788
130. Starr, R., et al. (1997) Nature 387, 917-921
131. Starr, R., and Hilton, D. J. (1998) The Int. J. Biochem. Cell Biol. 30, 1081-1085
132. Hilton, D. J., et al. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 114-119
133. Naka, T., et al. (1999) TIBS 24, 394-398
134. Kamura, T., et al. (1998) Genes & Development 12, 3872-3881
135. Matsumoto, A., et al. (1997) Blood 89, 3148-3154
136. Naka, T., et al. (1997) Nature 387, 924-929
137. Yoshimura, A., et al. (1995) EMBO J. 14, 2816-2826
138. Endo, T. A., et al. (1997) Nature 387, 921-924
139. Adams, T. E., et al. (1998) J. Biol. Chem. 273, 1285-1287
140. Hansen, J. A., et al. (1999) Mol. Endocrinol. 13, 1832-1843
141. Favre, H., et al. (1999) FEBS Lett. 453, 63-66
142. VanderKuur, J. A., et al. (1995) J. Biol. Chem. 270, 21738-21744
143. Ram, P. A., and Waxman, D. J. (1999) J. Biol. Chem. 274, 35553-25561
144. Yasukawa, H., et al. (1999) EMBO J. 18, 1309-1320
145. Narazaki, M., et al. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13130-13134
146. Kaelin, W. G., et al. (1998) Trends Genet. 14, 423-426
147. Conaway, J. W. (1998) Biochim. Biophys. Acta 1377, M49-M54
148. Stebbins, C. E., et al. (1999) Science 284, 455-461
149. Zhang, J.-G., et al. (1999) Proc. Natl. Acad. Sci. USA 96, 2071-2076
150. Lonergan, K. M., et al. (1998) Mol. Cell. Biol. 18, 732-741
151. De Sepulveda, P., et al. (2000) J. Biol. Chem. 275, 14005-14008
152. Verdier, F., et al. (1998) J. Biol. Chem. 273, 28185-28190
153. Ram, P. A., and Waxman, D. J. (2000) J. Biol. Chem. 275, 39487-39496
154. Callus, B. A., and Mathey-Prevot, B. (1998) Blood 91, 3182-3192
155. Yu, C.-L., and Burakoff, S. J. (1997) J. Biol. Chem. 272, 14017-14020
156. Kamizono, S., et al. (2001) J. Biol. Chem. Paper in Press
157. Murray, A. (1995) Cell 81, 149-152
158. Pagano, M. (1997) FASEB J. 11, 1067-1075
159. Hershko, A. (1997) Curr. Opin. Cell Biol. 9, 788-799
160. Huang, Y., et al. (1995) Science 270, 1828-1831
161. Haas, A. L., et al. (1995) J. Biol. Chem. 270, 9407-9412
162. Kishino, T., et al. (1997) Nature Genetics 15, 70-73
163. Matsuura, T., et al. (1997) Nature Genetics 15, 74-77
164. Strous, G. J., van Kerkhof, P., Govers, R., Rotwein, P., and Schwartz, A. L. (1997) J. Biol. Chem. 272, 40-43
165. Hicke, L. (1997) FASEB J. 11, 1215-1226
166. Gropper, R., et al. (1991) J. Biol. Chem. 266, 3602-3610
167. Schwartz, A. L., et al. (1992) Am. J. Physiol. 262, C1031-C1038
168.,Grimm, L. M., et al. (1996) EMBO J. 15, 3835-3844
169. Becker, K., et al. (1997) FEBS Lett. 412, 102-106
170. Drexler, H. C. A. (1997) Proc. Natl. Acad. Sci. USA 94, 855-860
171. Bonifacino, J. S. (1996) Nature 384, 405-406

172. Oh, C. E., et al. (1994) J. Neurosci. 14, 3166-3179
173. Kim, T. K., and Maniatis, T. (1996) Science 273, 1717-1719
174. Lu, Z. M., et al. (1998) Mol. Cell Biol. 18, 839-845
175. Jentsch, S., et al. (1990) Trends Biochem. Sci. 15, 195-198
176. Schauber, C., et al. (1998) Nature 391, 715-718
177. Ciechanover, A., et al. (1991) Proc. Natl. Acad. Sci. USA 88, 139-143
178. Jungmann, J., et al. (1993) Nature 361, 369-371
179. Michalek, M. T., et al. (1993) Nature 363, 552-554
180. Rock, K. L., et al. (1994) Cell 78, 761-771
181. Hilt, W., and Wolf, D. H. (1996) Trends Biochem. Sci. 21, 96-102
182. Lenk, S. E., et al. (1992) J. Cell Biol. 118, 301-308
183. Chau, V., et al. (1989) Science 243, 1676-1583
184. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761-807
185. Jentsch, S. (1992) Annu. Rev. Genet. 26, 179-207
186. Varshavsky, A. (1992) Cell 69, 725-735
187. Handley-Gearhart, P. M., et al. (1994) J. Biol. Chem. 269, 33171-33178
188. McGrath, J. P., et al. (1991) EMBO J. 10, 227-236
189. Cook, J. C. (1991) Biochem. Biophys. Res. Commun. 174, 564-571
190. Grenfell, S. J., et al. (1994) Biochem. J. 300, 701-708
191. Finley, D., et al. (1984) Cell 37, 43-55
192. Finley, D., and Chau, V. (1991) Ann. Rev. Cell Biol. 7, 25-69
193. Jentsch, S., et al. (1991) Biochim. Biophys. Acta 1089, 127-139
194. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425-479
195. Varshavsky, A. (1996) Proc. Natl. Acad. Sci. USA 93, 12142-12149
196. Scheffner, M., et al. (1993) Cell 75, 495-505
197. Huibregtse, J. M., et al. (1995) Proc. Natl. Acad. Sci. USA 92, 2563-2567
198. Rotin, D., et al. (2000) J. Membr. Biol. 176, 1-17
199. Plant, P. J., et al. (2000) J. Cell Biol. 149, 1473-1484
200. Staub, O., et al. (1997) EMBO J. 16, 6325-6336
201. Bonifacino, J. S. W., A. M. (1998) Annu. Rev. Cell. Dev. Biol. 14, 19-57
202. Kamynina, E., et al. (2001) FASEB J. 15, 204-214
203. Tyers, M., et al. (1999) Science 284, 901-904
204. Joazeiro, C. A. P., et al. (2000) Cell 102, 549-552
205. Borden, K. L. (2000) J. Mol. Biol. 295, 1103-1112
206. Fang, S., et al. (2000) J. Biol. Chem. 275, 8945-8951
207. Honda, R. Y., H. (2000) Oncogene 19, 1473-1476
208. Waterman, H., et al. (1999) J. Biol. Chem. 274, 22151-22154
209. Joazeiro, C. A., et al. (1999) Science 286, 309-312
210. Yokouchi, M., et al. (1999) J. Biol. Chem. 274, 31707-31712
211. Yang, Y., et al. (2000) Science 288, 874-877
212. Sudakin, V., et al. (1995) Mol. Biol. Cell 6, 185-198
213. King, R. W., et al. (1995) Cell 81, 279-288
214. Page, A. M., et al. (1999) Annu. Rev. Biochem. 68, 583-609
215. Deshaies, R. J. (1999) Annu. Rev. Cell Dev. Biol. 15, 435-467
216. Iwai, K., et al. (1999) Proc. Natl. Acad. Sci. USA 96, 12436-12441
217. Lisztwan, J., et al. (1999) Genes & Dev. 13, 1822-1833
218. Pause, A., et al. (1999) Proc. Natl. Acad. Sci. USA 96, 9533-9538
219. Kamura, T., et al. (1999) Science 284, 657-661
220. Lavah-Baratz, S., et al. (1995) Proc. Natl. Acad. Sci. USA 92, 9303-9307

221. Bai, C., et al. (1996) Cell 86, 263-274
222. Kitagawa, M., et al. (1999) EMBO J. 18, 2401-2410
223. Yaron, A., et al. (1998) Nature 396, 590-594
224. Margottin, F., et al. (1998) Mol. Cell 1, 565-574
225. Kaiser, P., et al. (2000) Cell 102, 303-314
226. Koegl, M. (1999) Cell 96, 635-644
227. Aravind, L., et al. (2000) Curr. Biol. 10, R132-R134
228. Meacham, G. C., et al. (2001) Nat. Cell Biol. 3, 100-105
229. Connell, C., et al. (2001) Nat. Cell Biol. 3, 93-96
230. Chung, C. H. B., S. H. (1999) Biochem. Biophys. Res. Commun. 266, 633-640
231. Wilkinson, K. D. (2000) Semin. Cell. Dev. Biol. 11, 141-148
232. D'Andrea, A., et al. (1998) Crit. Rev. Biochem. Mol. Biol. 33, 337-342
233. Lam, Y. A., et al. (1997) Nature 385, 737-740
234. Cadavid, A. L., Ginzel, A., and Fischer, J. A. (2000) Development 127, 17271736
235. DeMartino, G. N., et al. (1999) J. Biol. Chem. 274, 22123-22126
236. Kloetzel, P.-M. (2001) Nature Rev. 2, 179-187
237. Baumeister, W., et al. (1998) Cell 92, 367-380
238. Lowe, J., et al. (1995) Science 268, 533-539
239. Groll, M., et al. (1997) Nature 386, 463-471
240. Brannigan, J. A., et al. (1995) Nature 378, 416-419
241. Dodson, G., et al. (1998) Trend Biochem. Sci. 23, 347-352
242. Orlowski, M. (1990) Biochemistry 29, 10289-10289
243. Coux, O., et al. (1996) Annu. Rev. Biochem. 65, 801-847
244. Glickman, M. H., et al. (1998) Cell 94, 615-623
245. Braun, B. C., et al. (1999) Nature Cell Biol. 1, 221-226
246. Glickman, M. H., et al. (1999) Mol. Biol. Reprod. 26, 21-28
247. Strickland, E., et al. (2000) J. Biol. Chem. 275, 5565-5572
248. Coffino, P. (2001) Nature Rev. 2, 188-194
249. Lee, D. H., et al. (1998) Trend Cell Biol. 8, 397-403
250. King, R. W., et al. (1996) Science 274, 1652-1659
251. Townsley, F. M., et al. (1998) Trend Cell Biol. 8, 238-244
252. Pamer, E., et al. (1998) Annu. Rev. Immunol. 16, 323-358
253. Chen, Z. J., et al. (1998) In Ubiquitin and the Biology of the Cell ed. J. M. Peters, J. R. Harris, D. Finley, 303-322. New York: Plenum
254. Hochstrasser, M., et al. (1998) In Ubiquitin and the Biology of the Cell ed. J. M. Peters, J. R. Harris, D. Finley, 279-302. New York: Plenum
255. Fenteany, G., et al. (1995) Science 268, 726-731
256. Bogyo, M., et al. (1997) Biopolymers 43, 269-280
257. Dick, L. R., et al. (1996) J. Biol. Chem. 271, 7273-7276
258. Bogyo, M., et al. (1998) Chem. Biol. 5, 307-320
259. McCormack, T., et al. (1997) J. Biol. Chem. 272, 26103-26109
260. Vinitsky, A., et al. (1994) J. Biol. Chem. 269, 29860-29866
261. Brodsky, J. L., et al. (1997) Trends Cell Biol. 7, 151-156
262. Cresswell, P., et al. (1997) Curr. Biol. 7, R552-R555
263. Kawahara, H., et al. (1992) Dev. Biol. 151, 27-33
264. Amsterdam, A., et al. (1993) Proc. Natl. Acad. Sci. USA 90, 99-103
265. Palombella, V. J., et al. (1994) Cell 78, 773-785
266. Sears, C., et al. (1998) J. Biol. Chem. 273, 1409-1419
267. Lin, L., et al. (1996) Mol. Cell. Biol. 16, 2248-2254

268. Rapoport, T. A., et al. (1996) Annu. Rev. Biochem. 65, 271-303
269. Plemper, R. K., et al. (1997) Nature 388, 891-895
270. Gorlich, D., and Rapoport, T. A. (1993) Cell 75, 615-630
271. Sommer, T., and Jentsch, S. (1993) Nature 365, 176-179
272. Biederer, T., et al. (1996) EMBO J. 15, 2069-2076
273. Coppi, M. V., and Guidotti, G. (1997) FEBS Lett. 405, 281-284
274. Fisher, E. A., et al. (1997) J. Biol. Chem. 272, 20427-20434
275. Hampton, R. Y., et al. (1997) Proc. Natl. Acad. Sci. USA 94, 12944-12948
276. Ravid, T., et al. (2000) J. Biol. Chem. 275, 25840-35847
277. Dobson, C. M., and J., E. R. (1998) EMBO J. 17, 5251-5254
278. Payne, A. S., et al. (1998) Proc. Natl. Acad. Sci. USA 95, 10854-10859
279. Kopito, R. R. (1999) Physiol. Rev. 79, S167-S173
280. Shamu, C. E., et al. (1999) J. Cell Biol. 147, 45-58
281. Hicke, L., and Riezman, H. (1996) Cell 84, 277-287
282. Chen, Z. J., et al. (1996) Cell 84, 853-862
283. Hicke, L. (1999) Trend Cell Biol. 9, 107-112
284. Strous, G. J., and Govers, R. (1999) J. Cell Sci. 112, 1417-1423
285. Trowbridge, I. S. (1991) Curr. Opin. Cell Biol. 3, 634-641
286. Marks, M. S., et al. (1997) Trends Cell Biol. 7, 124-128
287. Roth, A. F., and Davis, N. G. (1996) J. Cell Biol. 134, 661-674
288. Galan, J. M., and Haguenauer-Tsapis, R. (1997) EMBO J. 16, 5847-5854
289. Moreau, V., et al. (1997) Mol. Biol. Cell 8, 1361-1375
290. Lucero, P., and Lagunas, R. (1997) FEMS Microbiol. Lett. 147, 273-277
291. Horak, J., and Wolf, D. H. (1997) J. Bacteriol. 179, 1541-1549
292. Kolling, R., and Hollenberg, C. P. (1994) EMBO J. 13, 3261-3271
293. Jeffers, M., et al. (1997) Mol. Cell Biol. 17, 799-808
294. Mori, S., et al. (1995) Biochem. Biophys. Res. Commun. 213, 32-39
295. Mori, S., et al. (1995) J. Biol. Chem. 270, 29447-29452
296. Doherty, F. J., et al. (1989) Biochem. J. 263, 47-55
297. Laszlo, L., et al. (1990) FEBS Lett. 261, 365-368
298. Paolini, R., and Kinet, J. P. (1993) EMBO J. 12, 779-786
299. Miyazawa, K., et al. (1994) Blood 83, 137-145
300. Cenciarelli, R., et al. (1992) Science 257, 795-797
301. Schild, L., et al. (1996) EMBO J. 15, 2381-2387
302. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) EMBO J. 16, 4851-4858
303. Fazioli, F., et al. (1993) Mol. Cell Biol. 13, 5814-5828
304. van Delft, S., et al. (1997) J. Cell Biol. 136, 811-821
305. van Delft, S., et al. (1997) J. Biol. Chem. 272, 14013-14016
306. Wang, Y., et al. (1996) J. Biol. Chem. 271, 17-20
307. Miyake, S., et al. (1998) Proc. Natl. Aca. Sci. USA 95, 7927-7932
308. Wang, Y., et al. (1999) J. Cell Biochem. 72, 119-134
309. Levkowitz, G., et al. (1998) Genes Dev. 12, 3663-3674
310. Roupas, P., and Herington, A. C. (1988) Mol. Cell. Endocrinol. 57, 93-99
311. Murphy, L. J., and Lazarus, L. (1984) Endocrinol. 115, 1625-1632
312. Terrell, J., et al. (1998) Mol. Cell. 1, 193-202
313. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. 18, 28-36
314. Goodman Jr., O. B., et al. (1996) Nature 383, 447-450
315. Cahoreau, C., et al. (1994) FEBS Lett. 350, 230-234

316. Mori, S., et al. (1992) J. Biol. Chem. 267, 6429-6434
317. Mimnaugh, E. G., et al. (1996) J. Biol. Chem. 271, 22796-22801
318. van Kerkhof, P., Govers, G., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575-1580
319. Pickart, C. M. (1999) Stress Proteins, 133-152
320. Goff, S. A., and Golberg, A. L. (1985) Cell 41, 587-595
321. Hershko, A., et al. (1984) J. Biol. Chem. 257, 13964-13970
322. Ciechanover, A., Finley, D., and Varshavsky, A. (1984) Cell 37, 57-66
323. Finley, D., et al. (1987) Cell 48, 1035-1046
324. Treger, J. M., et al. (1998) Mol. Cell Biol. 8, 1132-1136
325. Cheng, L., et al. (1994) Mol. Gen. Genet. 243, 358-362
326. Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 543-550
327. Kulka, R. G., et al. (1988) J. Biol. Chem. 263, 15726-15731
328. Shang, F., et al. (1997) J. Biol. Chem. 272, 23086-23093
329. Haas, A. L., and Bright, P. M. (1985) J. Biol. Chem. 260, 12464-12473
330. Carlson, N., et al. (1987) J. Cell Biol. 104, 547-555
331. Ramanathan, M., et al. (1999) NeuroReport 10, 3797-3802
332. Crawford, D. W., and Blankenhorn, D. H. (1991) Atherosclerosis 89, 97-108
333. Witztum, J. L., and Steinberg, D. (1991) J. Clin. Invest. 88, 1785-1792
334. Ames, N. B., et al. (1993) Proc. Natl. Acad. Sci. USA 90, 7915-7922
335. Jenner, P. (1994) Lancet 344, 796-798
336. Keller, J. N., et al. (1997) J. Neurochem. 69, 274-284
337. Sziraki, I., et al. (1998) Neurosci. 85, 1101-1111
338. Rao, G. N. (1996) Oncogene 13, 713-719
339. Gonzalez-Rubio, M., et al. (1996) Kidney Int. 50, 164-173
340. Rosette, C., and Karin, M. (1996) Science 274, 1194-1197
341. Knebel, A., et al. (1996) EMBO J. 15, 5314-5325
342. Abe, M. K., et al. (1998) Am. J. Respir. Cell Mol. Biol. 18, 562-569
343. Lee, S. R., et al. (1998) J. Biol. Chem. 273, 15366-15372
344. Shang, F., and Taylor, A. (1995) Biochem. J. 307, 297-303
345. Jahngen-Hodge, J., et al. (1997) J. Biol. Chem. 272, 28218-28226
346. Reinheckel, T., et al. (1998) Biochem. J. 335, 637-642
347. Chiacchia, K. B., and Drickamer, K. (1984) J. Biol. Chem. 259, 15440-15446
348. De Wit, R., et al. (2000) Free Rad. Biol. & Med. 28, 28-38

Chapter II

The Signal Transduction of the Growth Hormone Receptor Is Regulated by the Ubiquitin/Proteasome System and Continues After Endocytosis

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Abstract

The growth hormone receptor (GHR) intracellular domain contains all of the information required for signal transduction as well as for endocytosis. Previously, we showed that the proteasome mediates the clathrin-mediated endocytosis of the GHR. Here we present evidence that the proteasomal inhibitor MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. If proteasomal inhibitor was combined with ligand in an endocytosis-deficient GHR mutant, the same phenomena occurred indicating that proteasomal action on tyrosine dephosphorylation is independent of endocytosis. Experiments with a GHR truncated tail mutant (GHR1-369) led to a prolonged JAK2 phosphorylation due to the loss of a phosphatase-binding site. This raised the question of what happens to the signal transduction of the GHR after its internalization. Co-immunoprecipitation of GH-GHR complexes before and after endocytosis showed that JAK2 as well as other activated proteins are bound to the GHR not only at the cell-surface but also intracellularly, suggesting that the GHR signal transduction continues in endosomes. Additionally, these results provide evidence that GHR is present in endosomes both in its full-length and truncated form, indicating that the receptor is downregulated by the proteasome.

Introduction

The growth hormone receptor (GHR) is a member of the cytokine/hematopoietin receptor superfamily (for review see 1). Cytokines regulate different aspects of cellular growth, differentiation and activation and play a critical role in immune and inflammatory responses. In response to GH, two GHR polypeptides dimerize, turning on a cascade of events leading to signal transduction by activating gene transcription in the cell nucleus and, at the same time, downregulation and degradation of the receptor (2, 3).
One major characteristic of the cytokine receptor family is the absence of an intrinsic tyrosine kinase activity. Upon dimerization, the GHR recruits and activates JAK2, a member of the Janus family of cytosolic kinases (4-6). Once bound, the two JAK2 molecules are in opposition and can transphosphorylate each other. Subsequently, the receptor chains become tyrosine phosphorylated allowing them to interact with other intracellular signaling components (6). JAK2 acts via special signal transducers and activators of transcription proteins (STATs), which dimerize and translocate to the nucleus to convey the appropriate signal to specific regulatory DNA-response elements (7, 8). In addition, JAK2 activation by GH facilitates initiation of various pathways including the Ras, mitogen-activated protein kinase (MAPK), the insulin receptor substrate (IRS) and the phosphatidylinositol 3-kinase (PI-3K) pathway (9, 10). GH-induced activation of the JAK/STAT signal transduction pathway is both rapid and transient. The molecular mechanism of JAK deactivation is still poorly understood. Part of the dephosphorylation of the GHR has been previously attributed to the activation of the tyrosine phosphatase SHP-1 (11). This enzyme was found to interact with JAK2 and GH stimulates the catalytic activity of SHP-1 (11). Another candidate could be SHP-2 since it associates with the GHR, and binding to JAK2 has also been reported. However, no dephosphorylation of JAK2 by SHP-2 could be demonstrated (12). Recently, another negative regulatory pathway of the GH receptor signaling involving the SOCS (suppressor of cytokine signaling) proteins has been identified (13, 14). The SOCS proteins appear to form part of a negative feedback loop that regulates cytokine signal transduction. Their expression is rapidly induced by activation of the JAK/STAT pathway (15).
Another important system which downregulates the GHR is the ubiquitin-proteasome system. This system regulates the degradation of nuclear and cytosolic proteins via the proteasome (16). The target proteins are first tagged with ubiquitin molecules to form a polyubiquitin chain, which is specifically recognized by the multi-subunit proteasome complex, leading to their degradation. Proteasomes were found to be involved in regulating JAK/STAT pathways upon interleukin-2, -3 and erythropoietin stimulation (17-19). In the presence of specific proteasomal inhibitors, activation of both JAK and STAT molecules was sustained, although neither STAT nor JAK appeared to be ubiquitinated. These data indicate that protea-

somes are involved in the downregulation of the activation signals of specific cytokine receptors.
An important factor in GHR down-regulation is its endocytosis. In the presence of ligand GHR endocytoses rapidly via clathrin-coated pits (20), and its degradation occurs at least partially within the lysosome (21). The ubiquitin system is required for ligand-induced GHR internalization (14). In particular, the UbE motif within the GHR cytosolic tail is involved in both GHR ubiquitination and ligand-induced endocytosis (22). In a chinese hamster cell line carrying a temperature-sensitive E1 enzyme (ts20 cells), inactivation of E1 results in accumulation of non-ubiquitinated GHRs at the plasma membrane, while internalization of the transferrin receptor is unaffected (23). Recently, we showed that the proteasome is also involved in GHR downregulation (24). GHR internalization requires proteasomal action in addition to an active ubiquitin conjugation system. Specific proteasomal inhibitors block GH uptake of the full-length GHR, while a truncated receptor can endocytose undisturbed.
In this report, we address the role of proteasome-mediated protein degradation in modulating GHR/JAK2 activity following GH stimulation. We show that the proteasomal inhibitor MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. Furthermore, we observe that JAK2 is not only bound to the GHR at the cell-surface but also intracellularly, suggesting that the receptor and other signal transducing molecules are still active in endosomes.

Experimental procedures

Cells and Materials

Chinese hamster ts20 cells were stably transfected as described previously (23). Truncated receptors were constructed by introducing stop codons at various positions within the rabbit cDNA (25). These truncated GHR cDNAs were cloned in pcDNA3 (In Vitrogene Inc.) and transfected into ts20 cells, resulting in cell lines stably expressing receptors truncated at amino-acid residues 399 and 369. The internalization-deficient mutant GHR F327A was constructed by site-directed mutagenesis, cloned and transfected into ts20 cells as described before (25). Stable, geneticin-resistant transfectants were grown in Eagle's minimal essential medium (MEM-α) supplemented with 10% fetal bovine serum, penicillin and streptomycin, 4.5 g/liter glucose, and 0.45 mg/ml geneticin. For experiments, cells were grown in the absence of geneticin at approximately 70% confluence.
Antibody to GHR was raised against amino acids 271-381 of the cytoplasmic tail (anti-GHR(T)) as previously described (23). Antibody (Mab5) recognizing the lumenal part of the GHR was from AGEN Inc., Parsippany, NJ. Antiserum against JAK2 was raised in rabbits against a synthetic peptide corresponding to the hinge

region (amino acids 758-777) between domains 1 and 2 of murine JAK2. Polyclonal antibody against JAK2 and phosphotyrosine (4G10, anti-PY) were obtained from Upstate Bio-technologies Inc. (Lake Placid, NY). Antiserum against human GH was raised in rabbits. Commercial anti-GH was from DAKOPATTS. hGH was a gift of Lilly Research Labs, Indianapolis, IN. Culture medium, fetal calf serum (FCS) and geneticin were purchased from Gibco. MG132 was from CalBiochem.

Cell lysis, immunoprecipitation and immunoblotting

Cells, grown in 10-cm dishes, were first incubated for 2 hours at 30°C in FCS-free MEM-α- in presence or absence of 20 mM MG132. After hGH (8 nM) incubation, cells were lysed on ice in 0.6 ml of lysis mix containing 1% Triton X-100, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 50 mM NaF in PBS. The immunoprecipitations were performed in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 50 mM NaF in PBS. The lysates were cleared by centrifugation and incubated with GHR antiserum or JAK2 antiserum for 2 hours on ice. Protein A-agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immune complexes. The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. Immune complexes were analyzed by poly-acrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained using either Mab 4G10 (anti-PY), anti-GHR or commercial JAK2 antibody. After incubating the blots with rabbit anti-mouse IgG (RAMPO) or protein A conjugated to HRP, antigens were visualized using the ECL system (Boehringer Mannheim).

Co-immunoprecipitation of internalized proteins

Cells, grown on 10-cm dishes, were incubated 1 hour on ice in MEM-a, supplemented with 20 mM Hepes, and with 8 nM hGH. The cells were then washed once to remove unbound GH and incubated at 30°C for different periods of time. Cells were put on ice after which the cell- surface labeled GH was removed by two times 30 sec with acidic solution of 50 mM glycine, 150 mM NaCl pH 2.5. The cells were washed with PBS and lysed in 0.1% Triton X-100, 1 mM EDTA, 0.5% BSA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4 and 50 mM NaF in PBS. Immunoprecipitations were performed in the same buffer with GH antiserum for 2 hours on ice. The immune complexes were treated as above. The blots were immunostained using Mab 4G10 or JAK2 antibody.

For the acid wash control, a competition assay was performed by using unstimula-

ted lysates of GHR 1-369 to lyse GH-stimulated wtGHR cells that were treated as described above.

Results

Effect of specific proteasomal inhibitors on GHR phosphorylation

To determine whether the proteasome is involved in modulating GHR activity, we examined the effects of the proteasomal inhibitor MG132 on GH-induced GHR phosphorylation. Using ts20 cells stably transfected with wtGHR, the time course of tyrosine phosphorylation of the GHR was determined following GH stimulation in the presence and absence of MG132 (Fig. 1). In the absence of GH, no phosphorylated GHR was visible (Fig. 1A). Upon GH stimulation a broad 130-kDa band appeared, indicating the GHR tyrosine phosphorylation. The activity was maximal within 15 min and decreased thereafter. If MG132 was present, virtually no decrease of the GHR phosphorylation signal was observed even after two hours. Reblotting with an anti-GHR antibody showed equivalent amounts of total immunoreactive protein in all samples, indicating that MG132 had little effect on the steady-state level of the GHR (Fig. 1B). Since GH stimulation was continuous, it was not possible to discriminate the population of down-regulated receptors from newly synthesized receptors as done previously by a metabolically labeled pulse-chase experiment (24). Thus, prolonged phosphorylation of the receptor due to the presence of MG132 could account for the sustained presence of the receptor at the cell-surface.

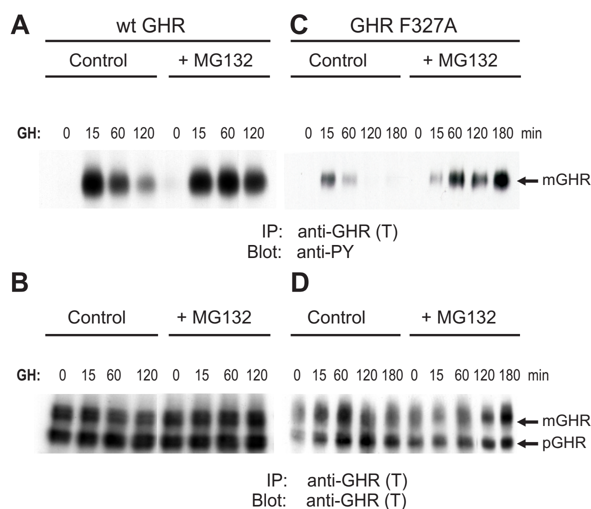

Fig. 1. **Effect of MG132 on GHR phosphorylation.** Cells were incubated for 2 hours at 30°C without (control) or with 20 mM MG132 and supplemented with 8 nM hGH for the time periods indicated. Cell lysates were prepared and subjected to immunoprecipitation (IP) of the GHR by using anti-GHR(T) and subjected to immunoblot analysis with an antibody against phosphotyrosine residues (anti-PY). **A**, ts20 cells expressing the wtGHR. **C**, ts20 cells expressing the GHR F327A. **B** and **D**, the PY blots were reblotted with anti-GHR(T). Arrows indicate mature (mGHR) and precursor (pGHR) forms of the GHR.

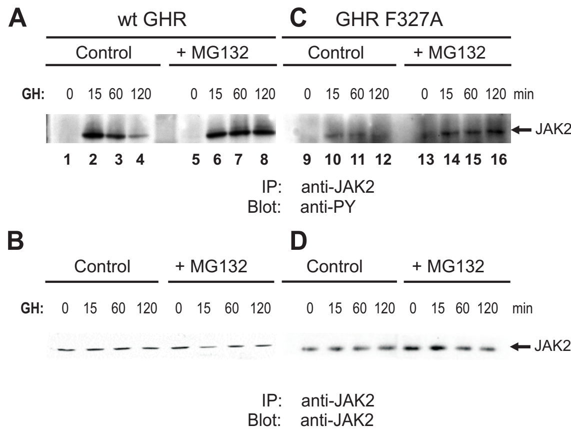

Fig. 2. **Effect of MG132 on JAK2 phosphorylation.** The procedure was as in Fig. 1, except that anti-JAK2 immunoprecipitations were subjected to an anti-PY immunoblot analysis. **A**, ts20 cells expressing the wtGHR. **C**, ts20 cells expressing the GHR F327A. In **B** and **D**, the resulting immunoprecipitates were reblotted with anti-JAK2.

The MG132 effect is not due to inhibition of internalization of GHR

Our previous results have shown that MG132 prevents internalization of the GHR (24). It is anticipated that a prolonged stay at the cell-surface might result in a prolonged phosphorylated state of the GHR and of JAK2. To test this, we used the GHR F327A transfected cells, which express receptors defective in internalization (25, 26). The kinetics of tyrosine phosphorylation/dephosphorylation of the GHR F327A were similar to the wtGHR (Fig. 1C), reaching a maximum after 15 min and decreasing to basal levels after 2 hours of GH treatment. However, in the presence of MG132, the level of tyrosine phosphorylation of the GHR F327A remained the same. Thus, down-regulation of the GHR phosphorylation depends on proteasomal action, and is not related to the GH-induced endocytosis.

GHR sustained activation is due to prolonged JAK2 phosphorylation

Proteasomal inhibitors prolong signaling of the interferon-gamma receptors after ligand stimulation, showing sustained tyrosine phosphorylation of both the receptors and JAK1/JAK3 (17). To determine whether the effect of MG132 on GHR phosphorylation is due to sustained activation of JAK2 kinase, anti-JAK2 immunoprecipitates were prepared from cell lysates and analyzed by immunoblotting with an antibody to phosphotyrosine. As shown in Fig. 2A, GH induced a transient phosphorylation of JAK2 with a maximum at 15 min in the absence of proteasomal inhibitor, declining to nearly basal levels after 2 hours. However, treatment of the cells with MG132 prevented the dephosphorylation of JAK2, correlating well with the sustained GHR activity (compare lanes 4 and 8). Reblotting with an anti-JAK2 antibody showed similar amounts of immunoreactive protein in all samples indicating that MG132 had little effect on the stability of the protein (Fig. 2B). Shorter

preincubation periods with MG132 were as effective in stabilizing the tyrosine phosphorylation of JAK2, suggesting that its mechanism of action is specific and not due to general cell toxic effects. The same was observed for the GHR F327A mutant (Fig. 2C and 2D). JAK2 activation was transient in this mutant, but as for the wtGHR, MG132 treatment prolonged JAK2 phosphorylation in a similar way. Taken together, these results demonstrate that MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. Thus, the proteasome plays a role in decreasing GHR signal transduction.

Possible role of proteasomes in modulating phosphatase activity

Previous reports have implicated JAK proteins in dephosphorylation by interaction with specific phosphatases (12,27-29). Hackett et al., using FDP-C1 cells, demonstrated that the region in the GHR cytosolic tail between 521 and 540 is required for inactivation of the JAK/STAT signaling cascade, possibly via the protein tyrosine phosphatase SHP-1 that acts as a negative regulator (11). However, SHP-1 does not seem to associate with the GHR. Also the tyrosine phosphatase SHP-2, another member of the protein-tyrosine phosphatase family that, unlike SHP-1, is ubiquitously expressed in vertebrate cells, was shown to form a complex with both the tyrosine phosphorylated receptor (GHR cytoplasmic domain residues 485-620) and JAK2 protein (30). We determined whether MG132 would also induce prolonged JAK2 phosphorylation upon GH treatment in a C-terminally truncated GHR. JAK2 was immunoprecipitated from GHR 1-399 and GHR 1-369 expressing cells and immunoblotted with antiphosphotyrosine antibodies for various times of GH treatment (Fig. 3A and C). In both cell lines, JAK2 showed tyrosine phosphorylation with no change during time. In accordance with the above-mentioned studies,

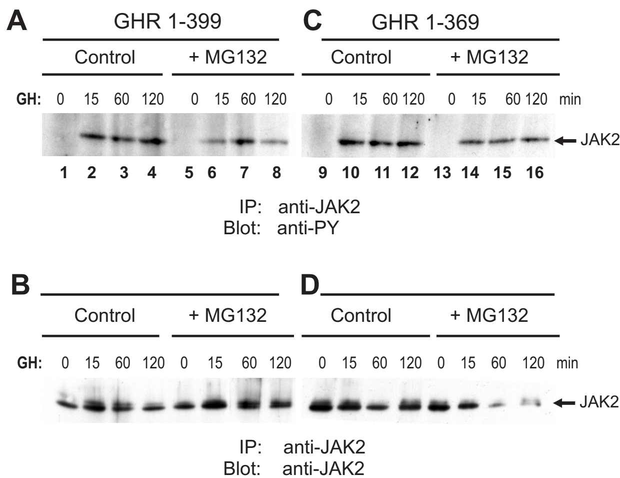

Fig. 3. **Effect of MG132 on JAK2 phosphorylation in truncated GHR?expressing cells.** Ts20 cells expressing GHR 1-399 (**A**) or GHR 1-369 (**C**) were incubated at 30°C without (control) or with 20 mM MG132 and supplemented with 8 nM hGH for the time periods indicated. The truncated forms of the GHR were immunoprecipitated with anti-GHR(T) and immunoblotted with anti-PY. Reblots with anti-GHR(T) are shown on **B** and **D**.

delayed dephosphorylation of the kinase was observed both in the presence and absence of MG132. Reprobing the blots with JAK2 antibody confirmed the presence of equal amounts of JAK2 protein in each sample (Fig. 3B and 3D). These results suggest that the activation of a negative regulator (SHP-1 or SHP-2) through distal GHR tail domains and further association with JAK2 might be the important factor responsible for down-regulating the GHR/JAK2 phosphorylation in a proteasome-dependent way. Thus, inhibition of the proteasome by MG132 inhibits the dephosphorylation of JAK2, resulting in prolonged activity of both JAK2 and GHR. However, MG132 had no effect on SHP-2 phosphorylation upon GH induction, or had any effect on SHP-2 binding to both GHR and JAK2 (results not shown).
As shown previously, proteasomal inhibitors do not affect internalization of GH via the GHR 1-369 but effectively block endocytosis of GHR 1-399 (24). As JAK2 phosphorylation is similar in both cell lines, the data implicate that signaling might continue after endocytosis.

JAK2 protein is bound to GHR in endosomes
Signaling via the GHR begins at the cell-surface. As demonstrated above using the endocytosis-defective GHR F327A cell line, the activation/deactivation (tyrosine phosphorylation/ dephosphorylation) cycle can be initiated and completed at the

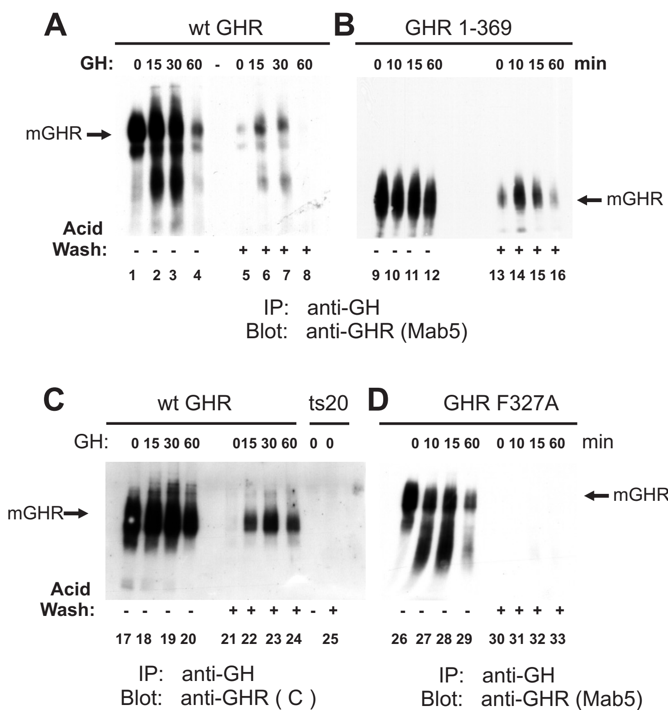

Fig. 4. **Acid-resistent GH-GHR complexes are found in the cell.** **A**, cells expressing full length wtGHR, **B**, GHR 1-369, and **D**, GHR F327A were incubated with 8 nM hGH on ice for 1 hour, followed by incubation at 30°C for the time periods indicated. The cells were then subjected or not to an acid wash procedure. The cell lysates were immunoprecipitated with ant-GH and blotted with anti-GHR (Mab5) or as in **C**, with anti-GHR (C) for cells expressing wtGHR.

cell-surface. The next question is whether signal transduction can continue after endocytosis. To address this, the activity of GHR has to be established after endocytosis. To accomplish this, we isolated GH-GHR complexes using anti-GH immunoprecipitation after acid treatment (Fig. 4). Dissociation of GH-GHR complexes does not occur at (endosomal) pH 5.5 (20). This indicates that GH remains complexed to its receptor, independent of its intracellular routing, unless it is localized to the lysosome. In that case, the ligand as well as the receptor is rapidly degraded (21). Treating living cells with buffers of pH higher than 2.5 showed that GH was not removed from GHR at the cell-surface, and only upon treatment with a buffer pH 2.5 did GH detach from the receptor, without interfering with the already internalized GH-GHR (results not shown). If no acidic treatment was performed, the total amount of wtGHR bound to GH co-immunoprecipitated during the different periods of time (Fig. 4A, lanes 1-4). The same was observed for the truncated GHR 1-369 (Fig. 4B, lanes 9-12) and GHR F327A (Fig. 4D, lanes 26-29). If the cells were kept on ice, acid treatment removed virtually all the GH from the cell-surface and hardly any GHR was detectable (Fig. 4, lanes 5, 13, 21 and 30). Upon incubation at 30°C, GH became acid-resistant indicating that GH-GHR complexes had entered the cells. Within 15 min, both wtGHR and GHR 1-369 were detectable inside the cells. Longer periods of GH treatment resulted in a decrease of GH-bound

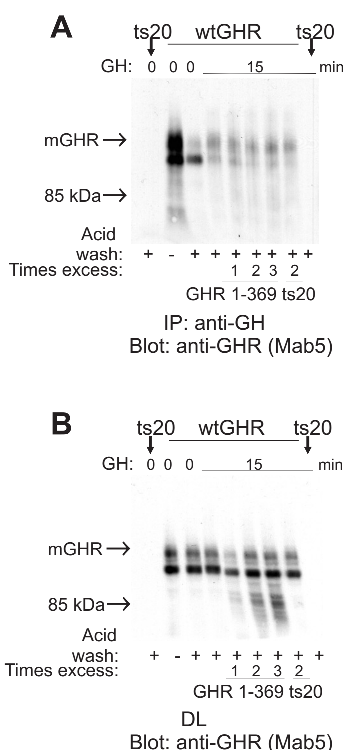

Fig. 5. **Acid-treatment control. A**, cells expressing wtGHR were treated with GH for 15 min and acid washed as previously, but lysis was performed in presence of cell extracts from unstimulated GHR 1-369 cells at 1, 2, or 3-fold excess concentrations of wtGHRs. The cell lysates were immunoprecipitated with anti-GH and blotted with anti-GHR (Mab5). **B**, total lysates were blotted with anti-GHR (Mab5).

internalized receptors in the endosomes (lanes 8 and 16). The GHR F327A was not observed inside the cells since internalization of this receptor is inhibited (lanes 30-33). In addition to the 130 kDa band of the GHR, a smear of bands (60-80 kDa) appeared which only reacted with Mab5 (Fig. 4A) and anti-GHR(T) (not shown) and not with anti-GHR C-terminal tail antibody (Fig. 4C). No degradation products were visible if GHR 1-369 was analyzed (not shown). These observations show that the partial degradation of the wtGHR starts from the C-terminus very soon after GH binding.

To exclude the possibility that during or after lysis GH is free to rebind endocytosed or cell-surface receptor, the same experiment for wtGHR was performed but excess of unstimulated GHR 1-369 lysate was added during lysis (Fig. 5). If free GH is available to react with wtGHR at the cell-surface or in endosomes, then an excess of GHR 1-369 will compete for binding to free GH. If this would be the case, then GH immunoprecipitates blotted with anti-GHR (Mab5) should present GH complexes with both wtGHR and GHR 1-369. As these receptors have different sizes but the same GH binding affinity, they can easily be distinguished by immunoblot. As observed on Fig. 5A, wtGHR cells were treated with GH for 15 min and acid treated. Addition of non-stimulated GHR 1-369 lysate in different concentrations, did not result in GH complexes containing the truncated receptor. Addition of excess of lysate of untransfected ts20 cells to the wtGHR expressing cells was also tested, with the same result. Fig. 5B shows total cell lysates blotted with anti-GHR (Mab5), indicating the amount of the truncated GHR 1-369 in the incubations. Performing the same experiment by lysing GH-treated GHR 1-369 cells in the presence of unstimulated wtGHR lysate, no GH-wtGHR complexes were detected (not shown).

We then addressed the question whether internalized GHR is able to bind JAK2. The same time course experiment was performed as in Fig. 4 and analyzed for JAK2 molecules (Fig. 6A). To measure the total amount of JAK2 bound to the GH-GHR complex, no acidification was performed (lanes 1-3). In wtGHR transfected cells upon acid wash, JAK2 co-immunoprecipitated with GH-GHR complexes after internalization (lanes 5 and 6). The same was observed in the GHR 1-369 mutant (lanes 7-9). A faster migrating background band reacted with anti-JAK2 after cell acidification, presumably due to proteolysis. As expected, the GHR F327A mutant did not show JAK2 binding after acidification. JAK2 was neither detectable in the anti-GH immunoprecipitates from untransfected ts20 cells (lane 13) nor unstimulated cells expressing the wtGHR, the GHR 1-369, and the GHR F327A (lanes 1, 4, 7 and 10), indicating the efficiency of the acid wash procedure. Similar amounts of JAK2 were found for the different cell lines as seen in Fig. 6B. These results show that JAK2 is bound to the GHR inside the cell, suggesting that the receptor is capable of signaling in endosomes.

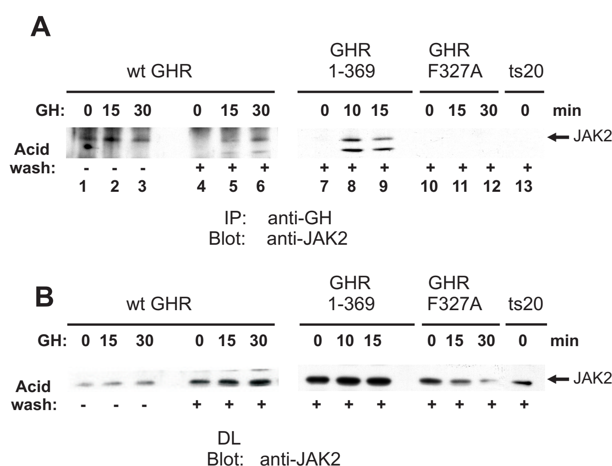

Fig. 6**. Acid-resistent GH-GHR complexes contain JAK2. A**, immunoprecipitates of GH-GHR complexes were blotted for anti-JAK2 for wtGHR, GHR 1-369, GHR F327A and ts20 untransfected cell lines. In **B**, the direct lysates were blotted for anti-JAK2 in the different cell lines as indicated.

GHR signaling continues inside the cell

To determine if other proteins attached to the GH-GHR in endosomes are phosphorylated, cells expressing wtGHR and GHR 1-369 were treated as described above and analyzed for phosphotyrosine positive proteins. As seen in Fig. 7A, GH induced the phosphorylation of a set of high molecular weight proteins. Upon GH removal from the cell-surface, the same set of proteins in the higher molecular weight range, attached to the internalized GH-GHR complex, were phosphorylated. Untransfected ts20 cells only resulted in a background pattern. In this molecular weight range both wtGHR and JAK2 proteins are possible candidates, consistent with the results presented above. Surprisingly, both wild type and truncated GHR 1-369 presented similar pattern of phosphorylated proteins. This can be explained by the fact that signaling proteins mainly interact with the membrane proximal region of the cytosolic tail of the GHR via JAK2. Although mainly high molecular weight proteins were phosphorylated, we cannot exclude that also lower molecular weight proteins were activated since the immunoprecipitation might be ineffective or the amount of molecules insufficient to allow their detection. Similar amounts of the receptor were found in each lane as observed in Fig. 7B.

Discussion

The ubiquitin-proteasome system plays an essential role in many cellular regulatory processes including cell cycle progression, DNA repair, transcriptional control and cell-surface-associated receptor endocytosis. In all these processes the ubiquitin-conjugating system targets ubiquitinated proteins to the proteasome for degra-

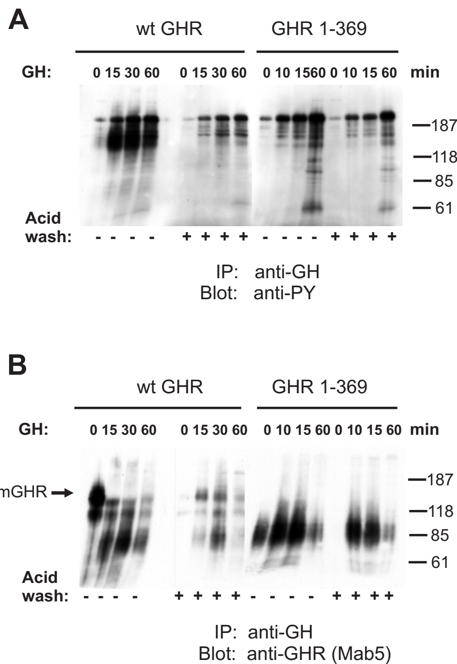

Fig. 7. **Phosphorylation of proteins attached via the GH-GHR complex after internalization.** The same procedure as in Fig 4. Immunoprecipi-tates of GH-GHR complexes from wtGHR, GHR 1-369 and untransfected ts20 cells were blotted with anti-PY, and control blots of direct lysates were immunoblotted with anti-GHR(T), as shown respectively in **A** and **B**.

dation. For the GHR, the ubiquitin system was found to be involved in GH-dependent endocytosis (31, for review see 32). In the present study, we demonstrate that the ubiquitin-proteasome system is involved in the down-regulation of GHR signal transducing events. Others have demonstrated that in several cytokine receptors the JAK/STAT pathway was downregulated by the proteasome. Both interleukin-2 and -3 and interferon receptor showed a prolonged JAK/STAT activation as well as other signaling molecules like MAP kinases, upon treatment with specific proteasomal inhibitors (18, 17). The studies described in this paper demonstrate a similar effect for the GHR. Using ts20 cells stably transfected with wtGHR, we show that, in the presence of the specific proteasomal inhibitor MG132, the phosphorylation/activation of both receptor and tyrosine kinase JAK2 are prolonged for long periods of GH induction. Furthermore, our data indicate that proteasomal action on signal transduction occurs at the cell-surface since signaling by the GHR F327A endocytosis-deficient mutant still depends on the proteasome for its down-regulation. These results support the notion that GHR/JAK2 signal down-regulation is not determined by endocytosis per se.
Which mechanisms underlie the down-regulation of the GHR and JAK2 proteins? Several reports have shown that tyrosine phosphatases are involved in the dephosphorylation of JAK proteins. Ligand-induced tyrosine phosphorylation/activation of JAK2 by eythropoietin receptor, induces binding of the protein tyrosine phos-

phatase SHP-1 to the cytoplasmic domain of the receptor. The recruitment of SHP-1 is accompanied by dephosphorylation of JAK2 and subsequent termination of eythropoietin-induced cellular proliferation (33, 34). A similar role for SHP-1 in mediating the down-regulation of JAK2 following stimulation of cells with GH has been proposed (11). Our results with GHR 1-399 and GHR 1-369 indicate that partial deletion of the C-terminal GHR tail leads to a prolonged JAK2 phosphorylation presumably due to loss of a negative regulator binding site and its consequent activation. This pattern of prolonged phosphorylation is similar to that of JAK2 in full length wtGHRs treated with MG132. One explanation might be that the phosphatase activity is modulated by proteasome function, perhaps by degrading an inhibiting complex in a similar manner as it occurs for the inhibitor of the transcription factor NF-κB (35). This would explain why in the presence of MG132, phosphatase inhibitors prevent dephosphorylation of the JAK2 by SHP-1, and thereby prolonging phosphorylation of both JAK2 and the GHR. In support of this model, SHP-1 degradation has been shown to be ubiquitin-dependent in mast cells (36), suggesting that the proteasome is involved in SHP-1 regulation. SHP-2, however, has been shown to interact directly with the tail of the GHR (residues 484-620) and associate with JAK2 and SIRPα1, a member of a family of transmembrane glycoproteins identified by its association to SH2 domain-containing SHP-2. In response to GH, JAK2 associates with SIRPα1 and rapidly stimulates tyrosine phosphorylation of both SIRPα1 and SHP-2, and enhances association of these two molecules (37). Recently, it was shown that SIRPα1 is acting as a negative regulator of GH signaling by its ability to bind SHP-2 (38). The proteasome could therefore play a role in SHP-2/SIRPα1 association and binding to JAK2. As SHP-2 is known to associate to other signaling molecules as IRS-1 (39) and p85-PI 3-K (40), future studies will indicate whether the MG132 effect on these molecules is directly related to SHP-2 activity. However, it cannot be excluded that activated JAK kinases themselves are subject to proteasome-mediated degradation. Support for this comes from a recent identified negative regulatory pathway of the GHR signaling involving the SOCS proteins. GH preferentially induces the rapid, transient expression of SOCS-3, a member of the SOCS family that is known to inhibit cytokine receptor signaling. Expression of other SOCS genes, SOCS-1, SOCS-2 and CIS, was also up-regulated by GH, although to a lesser extent than SOCS-3 and with different kinetics (14). Recently, it was shown that the highly conserved C-terminal homology domain of the SOCS proteins, termed the SOCS box, mediates interactions with elongins B and C, which in turn may couple SOCS proteins and their substrates to the proteasomal protein degradation pathway (41). How SOCS proteins inhibit JAK kinase activity is still not clear, but analogous to the family of F-box-containing proteins, SOCS box interaction with elongins B and C potentiates interaction with the proteasome complex. This would explain why, in presence of MG132, degradation of SOCS proteins and its associated proteins like JAK2 would

be prevented, and therefore induce sustained activation of JAK2 and, consequently the GHR. Evidence for a role of the ubiquitin-proteasome system in signal transduction came from experiments of Verdier and co-workers who showed that a Cis member of the SOCS family was ubiquitinated upon erythropoietin receptor activation (19). Thus, at least two mechanisms for the termination of the GHR phosphorylation might depend on proteolysis: the regulation of phosphatases and of the SOCS proteins.

Until now there is clear evidence that tyrosine kinase receptors, like the epidermal growth factor and the insulin receptor, continue to signal after endocytosis (42, 43). Our data with the GHR show that initiation as well as termination of its phosphorylation as well as of JAK2 can occur at the cell-surface. No evidence is available about signal transduction inside the cells. Combining an acid wash procedure with anti-GH immunoprecipitation we show that the GHR can induce a second round of signal transduction intracellularly. This is not unexpected because obviously GH keeps the two GHRs complexed after endocytosis. In this configuration JAK2 has high affinity for the complex and will either rebind (if it was removed during passage of the coated pits) or will keep its position on the dimerized tails once internalized. Although the amount of undegraded, endocytosed wtGHR is very small, the western blot signal of JAK2 complexed to the GH-GHR complexes is significant as compared to control (non acid-washed) cells. This indicates that the signaling capacity of GH-GHR complexes in endosomes is significant. The relevance for signal transduction in endosomes is not clear. It is possible that the signaling GHR complexes in endosomes differ from those at the cell-surface. This is not obvious from our data, because the SDS-PAGE patterns of phosphotyrosine-containing proteins of total and endocytosed GH complexes look very similar. Together, these observations indicate that GHR signal transduction continues or resumes after endocytosis and that the signals, regenerated at the two cellular locations, do not substantially differ.

Another point of discussion is the presence of truncated GHR originating from the wtGHR, not from the truncated GHR 1-369. Firstly, it is not clear where this process starts. Experiments with the GHR F327A show that it is ubiquitin system (UbE motif) independent, because it also occurs in this mutant GHR, and in presence of proteasome inhibitors its formation can still occur (unpublished results). Thus, the GHR is C-terminally truncated already at the cell-surface and the truncated GHR can endocytose, complexed to GH. It remains to be determined, whether this truncated GHR plays a role in signal transduction.

Both JAK2 and a multitude of other, mostly high molecular weight, proteins are activated and interact with both wtGHR and GHR 1-369 after GH induction in acid-wash treated cells. Co-immunoprecipitation of GH-GHR-JAK2 complexes, after uptake by the cells, shows that JAK2 is not only bound to the GHR at the cell-surface but also intracellularly, suggesting that the receptor and some of its signal

transducing molecules might still be active in endosomes. A smear of phosphorylated proteins attached to the GH-GHR complex inside the cells confirmed the receptor's capacity of signaling. Intracellularly, the pattern of phosphorylated proteins in wtGHR and GHR 1-369 is similar, providing both receptors comparable signaling capabilities.

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References:

1. Kelly, P. A., Ali, S., Rozakis, M., Goujon, L., Nagano, M., Pellegrini, I., Gould D., Djiane, J., Edery, M., Finidori, J., and Postel-Vinay, M. C. (1993 Recent Prog. Horm. Res. 48, 123-164
2. Argetsinger, L. S., and Carter-Su, C. (1996) Physiol. Rev. 76, 1089-1107
3. Mellado, M., Rodriguez-Frade, J. M., Kremer, L., von Kobbe, C., de Ana, A. M., Merida, I., and Martinez, A., C. (1997) J. Biol. Chem. 272, 9189-9196
4. Frank, S. J., Gilliland, G., Kraft, A. S., and Arnold, C. S. (1994) Endocrinol. 135, 2228-2239
5. VanderKuur, J. A., Wang, X., Zhang, L., Cambell, G. S., Allevato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1994) J. Biol. Chem. 269, 21709-21717
6. Carter-Su, C., and Smit, L. S. (1998) Recent Prog. Horm. Res. 53, 61-83
7. Smit, L. S., Meyer, D. J., Billestrup, N., Norstedt, G., Schwartz, J., and Carter--Su, C. (1996) Mol. Endocrinol. 10, 519-533
8. Wang, Y. D., and Wood, W. I. (1995) Mol. Endocrinol. 9, 303-311
9. Vanderkuur, J. A., Butch, E. R., Waters, S. B., Pessin, J. E., Guan, K. L., and Carter-Su, C. (1997) Endocrinol. 138, 4301-4307
10. Thirone, A. C., Carvalho, C. R. O., and Saad, M. J. A. (1999) Endocrinol. 140, 55-62
11. Hackett, R. H., Wang, Y. D., Sweitzer, S., Feldman, G., Wood, W. I., and Larner, A. C. (1997) J. Biol. Chem. 272, 111280-11132
12. Yin, T., Shen, R., Feng, G. S., and Yang, Y. C. (1997) J. Biol. Chem. 272, 1032-1037
13. Ram, P. A., and Waxman, D. J. (1999) J. Biol. Chem. 274, 35553-25561
14. Adams, T. E., Hansen, J. A., Starr, R., Nicola, N. A., Hilton, D. J., and Billestrup, N. (1998) J. Biol. Chem. 273, 1285-1287
15. Starr, R., Willson, T. A., Viney, E. M., Murray, L. J. L., Rayner, J. R., Jenkins,

B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., and Hilton, D. J. (1997) Nature 387, 917-921
16. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761-807
17. Yu, C. L., and Burakoff, S. J. (1997) J. Biol. Chem. 272, 14017-20
18. Callus, B. A., and Mathey-Prevot, B. (1998) Blood 91, 3182-3192
19. Verdier, F., Christien, S., Muller, O., Varlet, P., Yoshimura, A., Gisselbrecht, S., Lacombe, C., and Mayeux, P. (1998) J. Biol. Chem. 273, 28185-28190
20. Roupas, P., and Herington, A. C. (1988) Mol. Cell. Endocrinol. 57, 93-99
21. Murphy, L. J., and Lazarus, L. (1984) Endocrinol. 115, 1625-1632
22. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. 18, 28-36
23. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. 15, 3806-3812
24. van Kerkhof, P., Govers, G., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575-1580
25. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) EMBO J. 16, 4851-4858
26. Allevato, G., Billestrup, N., Goujon, L., Galsgaard, E. D., Norstedt, G., Postel-Vinay, M. C., Kelly, P. A., and Nielsen, J. H. (1995) J. Biol. Chem. 270, 17210-17214
27. You, M., Yu, D. H., and Feng, G. S. (1999) Mol. Cell. Biol. 19, 2416-2424
28. Fauman, E. B., and Saper, M. A. (1996) TIBS 21, 413-417
29. Neel, B. G., and Tonks, N. K. (1997) Curr. Op. Cell Biol. 9, 193-204
30. Kim, S.-O., Jiang, J., Yi, W., Feng, G. S., and Frank, S. J. (1998) J. Biol. Chem. 273, 2344-2354
31. Strous, G. J., van Kerkhof, P., Govers, R., Rotwein, P., and Schwartz, A. L. (1997) J. Biol. Chem. 272, 40-43
32. Strous, G. J., and Govers, R. (1999) J. Cell Sci. 112, 1417-1423
33. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) Cell 80, 729-738
34. Jiao, H., Berrada, K., Yang, W., Tabrizi, M., Platanias, L. C., and Yi, T. (1996) Mol. Cell. Biol. 16, 6985-6992
35. Yaron, A., Gonen, H., Alkalay, I., Hatzubai, A., Jung, S., Beyth, S., Mercurio, F., Manning, A. M., Ciechanover, A., and Ben-Neriah, Y. (1997) EMBO J. 16, 6486-6494
36. Piao, X., Paulson, R., van der Geer, P., Pawson, T., and Bernstein, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14665-14669
37. Stofega, M. R., Wang , H., Ullrich, A., and Carter-Su, C. (1998) J. Biol. Chem. 273, 7112-7117
38. Stofega, M. R., Argetsinger, L. S., Wang, H., Ullrich, A., and Carter-Su, C. (2000) J. Biol. Chem. In press.

39. Myers, J., M. G., Mendez, R., Shi, P., Pierce, J. H., Rhoads, R., and White, M. F. (1998) J. Biol. Chem. 273, 26908-26914
40. Gesbert, F., Guenzi, C., and Bertoglio, J. (1998) J. Biol. Chem. 273, 18273-18281
41. Zhang, J. G., Farley, A., Nicholson, S. E., Willson, T. A., Zugaro, L. M., Simpson, R. J., Moritz, R. L., Cary, D., Richardson, R., Hausmann, G., Kile, B. J., Kent, S. B. H., Alexander, W. S., Metcalf, D., Hilton, D. J., Nicola, N. A., and Baca, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2071-2076
42. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) Science 274, 2086-2089
43. Ceresa, B. P., Kao, A. W., Santeler, S. R., and Pessin, J. E. (1998) Mol. Cell. Biol. 18, 3862-3870

Chapter III

Growth Hormone Receptor Ubiquitination, Endocytosis and Degradation are Independent of Signal Transduction via JAK2

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Abstract

The ubiquitin-proteasome system is required in growth hormone receptor endocytosis. For cytokine receptors, which lack intrinsic tyrosine kinase activity, signal transduction is initiated by the activation of a member of the JAK family. Previously, we have shown that GHR and JAK2 tyrosine (de)phosphorylation are regulated via the ubiquitin system. In this study, we examined the role of JAK2-mediated signal transduction in GHR internalization and downregulation. Mutation of the attachment site for JAK2, box-1, in the GHR cytoplasmic tail resulted in the complete absence of GHR and JAK2 phosphorylation. This modification did not alter the rate and extent of receptor-bound GH internalization as compared to a functional GHR, nor did it change its turnover and transport to the plasma membrane. In addition, the receptor was still normally ubiquitinated and remained dependent on both an intact ubiquitin system and on proteasomal action for its internalization. Thus, GHR ubiquitination, endocytosis and degradation occur independently of GHR signal transduction via JAK2. We conclude that, while endocytosis and degradation require the ubiquitin system, they are independent of GHR signal transduction.

Introduction

GH regulates important physiological processes such as growth, metabolism and cellular differentiation. The actions of GH are mediated through activation of the GHR, a member of the cytokine/hematopoietin receptor superfamily with homologies defined in the extracellular domain and that lacks intrinsic tyrosine kinase activity in its intracellular domain (1-3). Upon GH binding, the dimerized complex associates with the tyrosine kinase JAK2, a member of the Janus family of cytosolic kinases (3-5). GH-dependent tyrosine phosphorylation of JAK2 itself, GHR, and of other cellular proteins depends on the receptor's ability to activate JAK2 (3-9). Biochemical evidence has shown that JAK2 activation by the GHR is essential for activating the STAT proteins, several proteins involved in the Ras/MAP kinase pathway, as well as the insulin receptor substrate proteins IRS-1 and IRS-2, which initiate the PI-3 kinase pathway (4, 9, 10, 11). So far, only the GH-dependent effect on calcium entry appears to involve mechanisms independent of JAK2 activation (12). Since a C-terminally truncated receptor, able to interact with and to activate JAK2, was unable to activate specific signaling molecules, like STAT5 (13), activation of JAK2 alone is insufficient to elicit all of the responses to GH, suggesting that such proteins are unlikely to be direct JAK2 substrates.
A proline-rich motif termed box-1, between amino-acids 297-311, is conserved within members of the cytokine family and is required for the association of JAK2 with GHR as well as GH-dependent activation of JAK2 (1, 4, 14). Studies using mutated box-1 regions of the GHR have shown that this region is intermediate in GH-dependent association and activation of JAK2 (4, 7). Although box-1 is sufficient to bind and activate JAK2, a maximal JAK2 activation requires downstream residues in the half-proximal part of the GHR cytoplasmic domain. This more distal region appears to stabilize the interaction between the receptor and JAK2. Within box-1, no specific residue seems, in itself, crucial for the association. Mutation of each individual proline residue nor simultaneous mutation of the first two prolines in box-1 did impair JAK2 association to the receptor. On the other hand, simultaneous mutation of the 4 prolines abolished the capacity of the receptor to interact and to activate JAK2, as well as other signaling proteins (4, 15, 16). Thus, the proline-rich region is critical for GH signal transduction.
After binding to its receptor, GH internalizes via clathrin-coated pits and is degraded in lysosomes (17,18). This process is regulated by both the ubiquitin system and the proteasome (19-22). For other signaling receptors containing tyrosine kinase activity, like the epidermal growth factor (EGF) (23) and G protein-coupled receptors (24, 25), it was shown that kinase activity is necessary for their maximal internalization rate. In the EGF receptor, mutation of its intrinsic tyrosine kinase activity abolished both signaling transduction and ligand-induced downregulation/endocytosis (26, 27), while other receptors (e.g. transferrin) were internalized through

coated pits without any known kinase requirement (23).
Until now a role for JAK2 kinase activity of the GHR in endocytosis has not been described. The role of box-1 in endocytosis is unclear and mutations in box-1 may lead to a block of GHR endocytosis (15). Recently, we observed that signalling of GHR/JAK2 is also regulated by the proteasome, as proteasomal inhibitor MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. If proteasomal inhibitor was combined with ligand in an endocytosis-deficient GHR mutant, the same phenomena occurred indicating that proteasomal action on tyrosine dephosphorylation is independent of endocytosis (28). To determine the role of JAK2-mediated signal transduction in GHR internalization and downregulation, we replaced the four proline residues in box-1 with alanines ($GHR_{4P\text{-}A}$). Modification of the box-1 resulted in the complete absence of GHR and JAK2 phosphorylation. We demonstrate that the $GHR_{4P\text{-}A}$ mutant behaves in a similar manner as the wtGHR. $GHR_{4P\text{-}A}$ presents presents the same internalization kinetics as wtGHR, it is ubiquitinated normally, and depends on an intact ubiquitin system as well as on the proteasome for its internalization.

Experimental Procedures

Cells and Materials

Chinese hamster ts20 cells were stably transfected with the full-length rabbit GHR cDNA sequence as described previously (19), as well as a cDNA encoding for a GHR mutation on box-1 (see mutagenesis and transfection). Due to a thermolabile ubiquitin-activating enzyme (E1), the ubiquitin conjugating system is inactive in these cells at the non-permissive temperature of 42^{0}C. Stable geneticin-resistant transfectants were grown in Eagle's minimal essential medium (MEMα) supplemented with 10% fetal bovine serum, penicillin and streptomycin, 3.5 g/liter glucose and 0.45 mg/ml geneticin. For experiments, cells were grown in the absence of geneticin to approximately 80% confluence. Anti-GHR antiserum was raised against amino acids 271-381 (anti-GHR(T)) of the cytoplasmic tail as previously described (19). Antibody recognizing the lumenal part of the GHR (Mab5) was from AGEN Inc., Parsippany, NJ. Antiserum against JAK2 was raised in rabbits as described in (29). Polyclonal antibody against JAK2 and phosphotyrosine (4G10, anti-PY) were obtained from Upstate Bio-technologies Inc. (Lake Placid, NY). GH antiserum was from DAKOPATTS and antiserum specific for protein-ubiquitin conjugates was a generous gift from Dr. A. Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). hGH was a gift of Lilly Research Labs, Indianapolis, IN. Culture medium, fetal calf serum (FCS) and geneticin were purchased from Gibco. MG132 was purchased from CalBiochem.

Mutagenesis and transfection
The signaling-deficient mutant GHR_{4P-A} was constructed by QuickChange mutagenesis (Stratagene), using a 5' oligonucleotide and a 3' oligonucleotide containing the unique restriction site KpnI. This GHR cDNA was cloned into pcDNA3 vector (Invitrogen), sequenced and transfected into ts20 cells at 30% confluence using the calcium phosphate transfection method. After transfection, individual clones were selected in geneticin based on the amount of GH binding and by immunoblotting cell extracts with an antibody to the N-terminus of the GHR.

GH binding and internalization
^{125}I-labeled hGH (^{125}I-GH) was prepared by using chloramine T (19). Cells were grown in 35-mm dishes, washed and incubated with MEMα supplemented with 20 mM Hepes (MEMα/Hepes) and 0.1% bovine serum albumin (BSA) for 1 hour at 30^{0}C, and further incubated for 1 hour on ice with 8 nM ^{125}I-GH in MEMα/Hepes, in the absence or presence of excess unlabeled GH. The cells were washed free of unbound GH and incubated from 0 to 1 hours at 30^{0}C. The cells were washed with ice-cold PBS, membrane-associated GH was removed by acid wash (0.15 M NaCl, 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. The cell extracts were counted in a LKB gamma counter.

Microscopy
Cy3-GH was prepared using a FluoroLink Cy3 label kit according to the supplier's instructions (Amersham Pharmacia Biotech.). Transfected ts20 cells, grown on coverslips, were incubated for 1 hour in MEMα/Hepes at either 30^{0}C or at the non-permissive temperature of 42^{0}C, and then incubated for 30 min in the presence of Cy3-GH (1 μg/ml). If necessary, MG132 (20 μM) dissolved in ethanol, was added 1 hour before the start of the experiment. Cells were washed with PBS to remove unbound label and fixed for 2 hours in 3% paraformaldehyde in PBS. After fixation, the cells were embedded in Mowiol, and confocal laser scanning microscopy was performed using a Leica TSC 4D system.

Cell lysis, immunoprecipitation and western blotting
Cells, grown in dishes, were lysed on ice in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were cleared by centrifugation. In ubiquitin blotting experiments, the cells were lysed in boiling lysis buffer containing 1% SDS in PBS in order to minimize isopeptidase activity. The lysate was heated at 100^{0}C for 5 min, sheared to break all DNA, and centrifuged for 5 min at 10,000 x g. Immunoprecipitations were carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin

(BSA), 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. Reactions were incubated with GHR(T) or JAK2 antiserum for 2 hours at 0^0C. Protein A-agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immuno-complexes. The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. For co-immunoprecipitations, cells were lysed in 0.1% Triton X-100, 0.5% BSA, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were further incubated with GH antibody for 2 hours and the immuno-complexes were isolated through protein A-agarose beads. All immuno-complexes were analyzed through 7.5% polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained with either Mab 4G10 (anti-PY), anti-ubiquitin, anti-GHR, or commercial JAK2 antibody. After incubating the blots with rabbit anti-mouse IgG (RAMPO) or protein A conjugated to HRP, antigens were visualized through the ECL system (Boehringer Mannheim).

Metabolic labeling

For metabolic labeling, the cells were incubated in methionine-free MEM for 30 min and then [^{35}S]methionine (3.7 MBq/ml TRAN-^{35}S labelTM, 40 TBq/mmol, ICN, Costa Mesa, CA) was added and labeled for 10 min at 30^0C in a CO_2-incubator. The radioactivity was chased for different times with or without 8 nM GH in the presence of MEMα containing 0.1 mM unlabeled methionine and 0.1% BSA. The radioactivity was determined using a StormTM imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image QuaNT software version 4.2a.

Results

JAK2 binding and activation is absent in a box-1 mutated GHR

To determine whether activation of the GHR and downstream signaling components are involved in GH-dependent endocytosis, the 4 proline residues between amino-acids 280-287 in box-1 of the cytosolic tail of the GHR were mutated to alanines (GHR_{4P-A}). An intact box-1 is required for the association of JAK2 with GHR and GH-dependent activation of JAK2 (4, 7). A ts20 cell line stably expressing GHR_{4P-A} with approximately the same number of GH binding sites at the cell-surface as the wild-type GHR ts20 cell line was used in this experiment. Immunoblot analysis of cellular extracts from both cell lines demonstrated that the mutant receptor expressed a GHR protein of the expected molecular size and that the level of total cell expression was comparable (Fig. 1A). To ascertain that the GHR_{4P-A} mutant was unable to respond to GH, the tyrosine phosphorylation of the GHR was

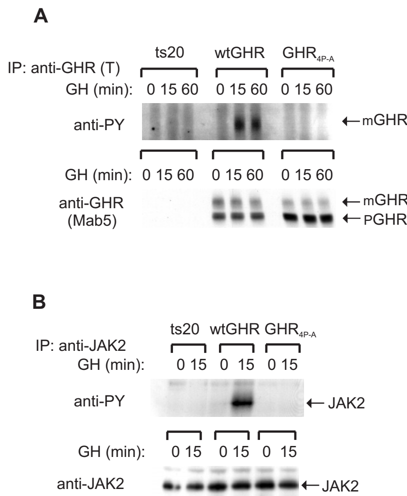

Fig. 1. **Box-1 is required for jAK2 binding and activation**
Untransfected ts20, ts20 cells expressing the wtGHR and GHR_{4P-A} cell lines were stimulated with 8 nM hGH at 30°C for the time periods indicated. Cell lysates were prepared and subjected to immunoprecipitation (IP) of the GHR by using anti-GHR(T) (A) or of JAK2 by using anti-JAK2 serum (B) and then subjected to immunoblot analysis with an antibody against phosphotyrosine residues (anti-PY). The PY blots were reblotted with anti-GHR(Mab5) or anti-JAK2, respectively. Arrows indicate mature (mGHR) and precursor (pGHR) forms of the GHR.

assayed. As shown in Fig. 1A, no PY signal was detectable on the 130 kDa GHR_{4P-A} mutant upon GH stimulation, in contrast to wtGHR expressing cells. As a negative control, untransfected ts20 cell lines were used. Similarly, JAK2 did not become tyrosine phosphorylated upon GH in cells expressing the GHR_{P-A} (Fig. 1B). Box-1 is, therefore, essential for activation of both JAK2 and GHR, confirming previously published results (4, 7). To test the capacity of the GHR_{4P-A} to activate signaling molecules other than JAK2, a co-immunoprecipitation of GH-GHR complexes was performed and blotted for phosphotyrosine proteins (Fig. 2). As expected, the inactive GHR_{4P-A} did not display the same phosphorylated bands as seen for the wtGHR, indicating that, indeed, the mutant receptor lacked signaling activity.

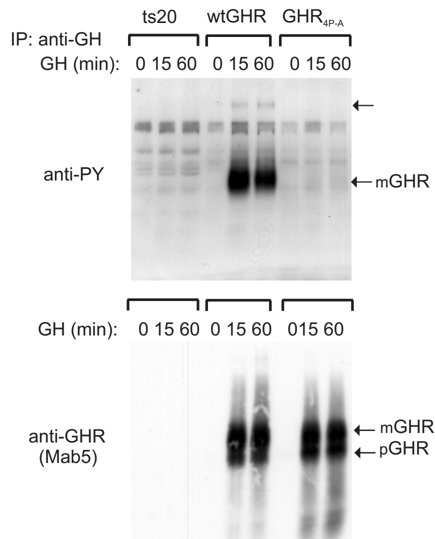

Fig. 2. **Tyrosine phosphorylation capacity of GHR_{4P-A} cells.**
Ts20 cells and cells expressing wtGHR and GHR_{4P-A} were incubated at 30°C with or without 8 nM hGH for the time periods indicated. Cells were lysed and GH-GHR complexes were co-immunoprecipitated with an antibody against GH (anti-GH), blotted with anti-PY (upper panel) and reblotted with anti-GHR (Mab5) (lower panel). Arrows indicate mature (mGHR) and precursor (pGHR) forms of the GHR.

JAK2 tyrosine kinase activity is not required for GH-dependent internalization of GHR.

To determine whether JAK2 tyrosine kinase activity is required for ligand-induced internalization, the uptake of ^{125}I-GH was performed in cell lines expressing either wtGHR or the GHR_{4P-A} mutant. As seen in Fig. 3, the percentage of internalization reached 50% after 30 min of GH induction for the wtGHR. In two independent cell lines, the rate of GH internalization for the box-1-mutated GHR was similar or higher than that of wild-type receptors. In order to exclude a possible contribution of receptor recycling, GH internalization was measured during the first 10 min of GH uptake and gave similar results for both types of receptors (not shown). In addition, the extent and rate of GH degradation were unaltered, indicating that JAK2 activation and GHR phosphorylation play no role in the transport of the ligand to the lysosomes. GHR_{4P-A} internalization bound GH as efficiently as wtGHR, indicating that tyrosine phosphorylation of the GHR is not required for GH-dependent GHR internalization.

To approach the same question in a different way, a fibroblastoma cell line deficient in JAK2 (γ2A) and its parental cell line containing JAK2 (2C4) transiently transfected with wtGHR were also tested for GH-uptake. Both cell lines showed the same endocytosis rates of ^{125}I-GH as observed for the GHR_{4P-A} mutant (not shown).

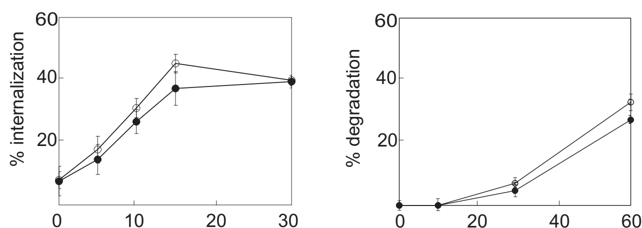

Fig. 3. **Internalization kinetics of wtGHR and GHR_{4P-A}.**
GH internalization was quantitated by [^{125}I]GH uptake. WtGHR cells (closed symbols) and GHR_{4P-A} (open symbols) were incubated with 8 nM [^{125}I]GH in the absence or presence of excess unlabeled GH for the indicated periods of time at 30^{0}C. Specific internalized [^{125}I] GH was expressed as the percentage of total radioactivity. TCA-soluble radioactivity in the incubation medium was determined and expressed as the percentage of total cell-associated and TCA-soluble label at 60 min.

Box-1 is not involved in GHR turnover

Next, we assessed the life-cycle of both the wtGHR and the GHR_{4P-A} mutant at 30^{0}C through pulse-chase labeling with [^{35}S]methionine (Fig. 4). The wtGHR is synthesized as an 110 kDa glycoprotein precursor and thereafter converted to an 130 kDa mature species. For both the wtGHR and the GHR_{4P-A}, the mature form was detectable at 20 min of chase and is maximal at 60 min, whereafter rapid degradation occurred (Fig. 4 A and B). These results demonstrate that both receptors have

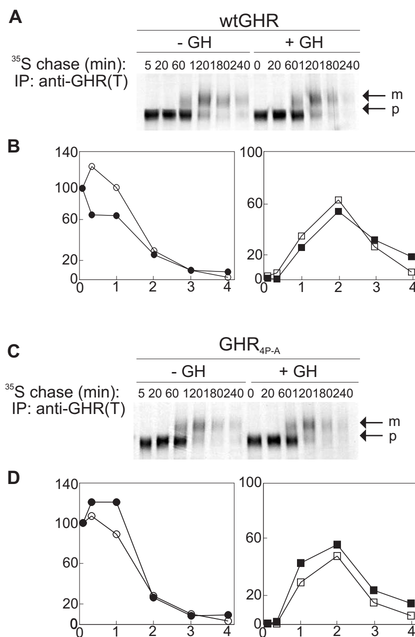

Fig. 4. **Pulse-chase labeling with [^{35}S]methionine of both wtGHR and GHR_{4P-A}.**
WtGHR and GHR_{4P-A}-expressing cells were pre-incubated with methionine-free MEMα for 30 min, labeled with [^{35}S]methionine for 10 min at 30^{0}C and chased in the absence of radioactivity in MEMα supplemented with 0.1% BSA, 100 μM methionine with or without 8 nM GH for the indicated times. Cell lysates were immunoprecipitated with anti-GHR antibody (anti-GHR(T)). (**A**), wtGHR and (**C**), GHR_{4P-A}; P, precursor GHR (110 kDa); m, mature GHR (130 kDa). Radioactivity was quantified by using ImageQuant (Molecular Dynamics) and expressed as a percentage of the radioactivity incorporated in the precursor GHR after the pulse labeling during time. **B,** precursor wtGHR is represented in absence of GH by closed circles and in presence of GH by open circles. Mature wtGHR is represented in absence of GH by closed squares and in presence of GH by open squares. **D**, precursor GHR_{4P-A} is represented in absence of GH by closed circles and in presence of GH by open circles. Mature GHR_{4P-A} is represented in absence of GH by closed squares and in presence of GH by open squares.

a fast turnover with a half-life of 45-60 min and that maturation and transport to the plasma membrane were not affected by a mutated box-1 in the GHR. To determine whether GH affects receptor turnover, the same experiment was performed in the presence of GH (Fig. 4, C and D). GH slightly accelerated degradation of the wtGHR mature form (Fig. 4C), as compared to GHR_{4P-A} (Fig. 4D).

Box-1 and ubiquitin/proteasome system-dependent internalization
Using ts20 cell lines, we showed previously that an active ubiquitin/proteasome system is essential for wtGHR internalization (19, 22). To elucidate this further, immunofluorescence studies were performed following the uptake of cy3-labeled GH for 30 min (Fig. 5). As a control, wtGHR cells were used (Fig. 5, A, B, C), clearly demonstrating an inhibition of internalization upon MG132 or 42^{0}C pre-incubation, similar to previous results (19, 22). For the GHR_{4P-A} mutant cells (Fig. 5, D, E, F), both treatments induced similar inhibitory effects as for the wtGHR. Fig. 5D confirms the results presented in Fig.3, clearly showing a normal ligand internalization staining pattern. Under conditions where either the proteasomes were inhibited (MG132) or the ubiquitin system was blocked (42^{0}C), cy3-GH-dependent GHR_{4P-A} mutant internalization was inhibited. To test whether kinase activity plays a role in GHR internalization, both cy3-GH and Texas Red-labeling

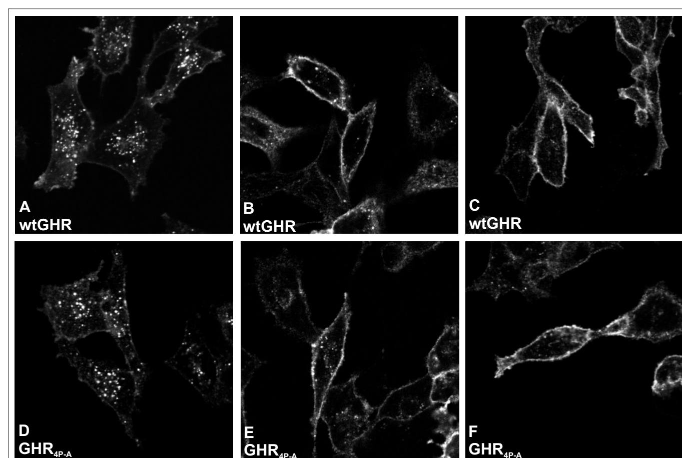

Fig. 5. **Visualization of Cy3-GH uptake by confocal microscopy**
Ts20 cells, expressing WtGHR (**A**-**C**) or $GHR_{4P\text{-}A}$ (**D**-**F**) were incubated for 1 hour at 30 (**A** and **D**) or 42^0C (**C** and **F**) or incubated with 20 µM MG132 at 30^0C (**B** and **E**). Cy3-GH was then added for 30 min, the cells were washed, fixed, and the fluorescence was visualized by confocal microscopy.

transferrin, used to control cellular transport, were measured in wtGHR cells treated in absence or presence of the kinase inhibitor staurosporine (not shown). Indeed, staurosporine had no effect on the internalization of either ligand, suggesting that tyrosine kinase activity is not essential for GHR endocytosis.

Ubiquitination of the GHR is independent of its phosphorylation and signal transduction
To determine whether GHR signaling is involved in receptor ubiquitination, we tested whether the $GHR_{4P\text{-}A}$ mutant was ubiquitinated upon GH stimulation. As shown in Fig.6, in the presence of GH, wtGHR was ubiquitinated at the permissive temperature but not ubiquitinated at the non-permissive temperature of 42^0C. Similar results were obtained for the $GHR_{4P\text{-}A}$. Thus, the ubiquitin-proteasome system does not require JAK2 action nor its presence on the dimerized GHR for its action to stimulate receptor endocytosis. In conclusion, GHR signal transduction via JAK2 is independent of ubiquitination, endocytosis and degradation.

Discussion

The GHR is a member of the cytokine/hematopoietin receptor superfamily, defined on the basis of a limited amino acid homology. In the intracellular domain of several members of this superfamily situated close to the plasma membrane, a proline-rich motif eight amino acids residues long, referred to as box-1, has been recognized as being involved in signal transduction (4, 7). The association of JAK2 to box-1 of the GHR, activation of the dimerized JAK2 kinase, and JAK2 autophosphorylation are all early steps in the signaling pathway (6). The central role of JAK2 in GH signaling is evident from studies using mutated GHRs which failed to bind or activate JAK2 and downstream effectors like SHC (Src homology (SH)-2 con-

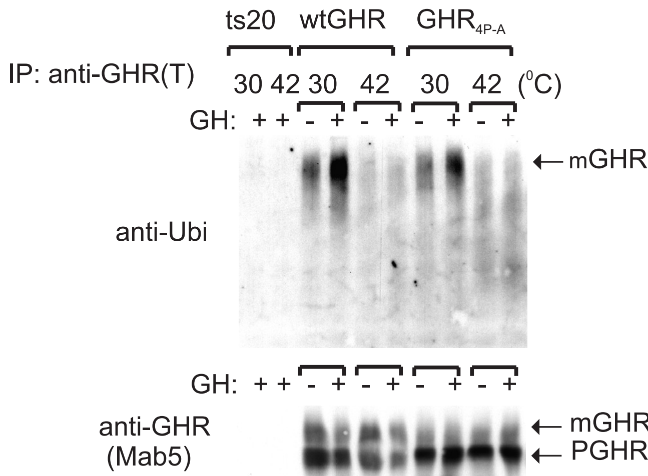

Fig. 6. **Effect of box-1 mutation in the GHR on ubiquitination.** Cells expressing wtGHR and $GHR_{4P\text{-}A}$ were incubated for 1 hour at 30 of 42⁰C and treated with 8 nM GH for 30 min. After lysis, GHR was immunoprecipitated with anti-GHR (T) and analyzed by Western blotting using an anti-ubiquitin conjugate serum (anti-Ubi). As a control, untransfected cells (ts20) were subjected to the same procedure. A reblot with anti-GHR (Mab5) is shown on the lower panel.

taining protein), MAP, STAT, and IRS (4, 9, 10, 11, 30).
The results of our experiments shown in Fig. 1, confirm that JAK2 can be specifically tyrosine phosphorylated in response to GH. Mutation of the four prolines of box-1 region at the membrane proximal domain of the cytoplasmic tail of the GHR abrogated JAK2 association with the GHR cytoplasmic domain as previously demonstrated (4). Although mutation of the box-1 region in the GHR did not affect the ability of the altered receptor to bind GH at the cell-surface, it dramatically affected the receptor's capacity to couple GH binding to JAK2 activation and consequently of other possible signaling molecules (Fig. 2). Interestingly, the biological responses of the GHR expressed in a variety of cell types (i.e. activation of MAP kinase, activation of c-fos gene expression, increased protein synthesis, lipid synthesis, and cellular proliferation), have not been observed for the GHR mutant of the box-1 region (30, 31, 32).
In contrast to what has been found with other families of signal-transducing growth factor receptors (e.g., EGF receptor), GH-stimulated JAK2 kinase activity and tyrosine phosphorylation of the GHR itself were not required for efficient GH-dependent internalization of the GHR (Fig. 3). A previous report suggested that mutations in box-1 may lead to a block of GHR endocytosis (15). In this study, the specific initial rate of internalization of the $GHR_{4P\text{-}A}$ appears to be nearly the same as for normal receptor. Furthermore, as observed in Fig. 3, GH is still normally targeted for degradation in $GHR_{4P\text{-}A}$, indicating that transport to the lysosome is not affected in box-1-defective receptors. The $GHR_{4P\text{-}A}$ turnover also does not differ from the wtGHR, suggesting that, once-internalized, intracellular sorting destinations of inactive receptor isoforms do not differ from those of functional receptor isoforms. However, upon GH stimulation, wtGHR mature form showed relatively faster degradation, while addition of GH had no such clear effect on the turnover of the

$GHR_{4P\text{-}A}$ (Fig. 4). This result suggests that GH-dependent wtGHR accelerated degradation is mediated through a JAK2-dependent process that is absent in $GHR_{4P\text{-}A}$, indicating that signal transduction is contributing to a faster endocytosis/degradation of the GHR. This might be due to the fact that the signal transduction pathway via STAT or MAP kinase stimulates the clathrin-mediated endocytosis, a phenomenon known from the EGF receptor studies (33).

Previously, we showed that the ubiquitin system is required for ligand-induced GHR internalization (19). In particular, a specific 10 amino-acid sequence between amino acid 323 and 332 termed the UbE motif within the GHR cytosolic tail is involved in both GHR ubiquitination and ligand-induced endocytosis (21). GHR internalization requires the recruitment of the ubiquitin conjugation system to the UbE motif. Recently, we showed that the proteasome is also involved in GHR downregulation (22). Specific proteasomal inhibitors block GH uptake of the full-length GHR, while a truncated receptor can endocytose undisturbed. Using ts20 cells, we determined whether the endocytosis of an inactive GHR is still inhibited upon inactivation of the ubiquitin system or upon inhibition of the proteasome with MG132. Our results show that at the non-permissive temperature, with an impaired ubiquitin system, $GHR_{4P\text{-}A}$ internalization was still inhibited as previously observed for the wtGHR (Fig. 5) (19). Thus, GHR/JAK2 signal transduction plays no role in the binding of the E2/E3 enzymes, members of the ubiquitin conjugation system, to the receptor (Fig. 3). Similarly, the proteasomal inhibitor MG132 prevented $GHR_{4P\text{-}A}$ endocytosis as previously determined for the wtGHR (Fig. 5) (22). This suggests that the motif at the GHR tail responsible for the proteasome action (amino acids 369-399) (22) does not interact with signaling proteins nor is such an interaction or phosphorylation itself important for the proteasome action. Furthermore, both wtGHR and mutated receptor are ubiquitinated upon GH binding (Fig. 6). Together, these results demonstrate that the molecular mechanism which underlies the ubiquitin-proteasome system-dependent endocytosis of the GHR does not require the presence nor the activity of JAK2 and its signalling molecules.

In a recently published work, we showed that proteasomes are involved in the downregulation of the GHR activation signals (28). The endocytosis-deficient receptor GHR F327A, was still able to become phosphorylated and to induce signal transduction. Surprisingly, its signal transduction was still downregulated at the cell-surface, indicating that it occurs independent of endocytosis. However, proteasomal inhibitors prevented GHR and JAK2 dephosphorylation at the cell-surface. We concluded that the ubiquitin-proteasome system is a regulator of JAK2 signal transduction, probably via suppressors of cytokine signalling (SOCS). Thus, the ubiquitin-proteasome system independently regulates the signal transduction capacity of the GHR in two ways: first, it determines the rate of endocytosis via the UbE-motif in the GHR tail, and second, it determines the signaling time via JAK2 and SOCS. Both mechanisms appear to be independently regulated by the same sys-

tem, probably via completely different E2/E3 enzyme systems.
In other systems, e.g. the EGF receptor, association of the adapter protein Grb2 is required for receptor endocytosis. Grb2 associates with the cytoplasmic tail of the EGF receptor after stimulation by EGF, leading to activation of the Ras/MAPK signalling pathway. Grb2 also indirectly associates with the GHR via SHC in the region that maps to the distal cytoplasmic tail (amino acids 454-620) (35). Cells expressing truncated receptors that lack this domain and therefore unable to associate with Grb2, can internalize the truncated receptor as efficiently as cells containing wtGHR. Thus, in addition to the dispensability of JAK2 activity and receptor tyrosine phosphorylation, recruitment of Grb2 to the receptor and activation of the MAPK pathway is also not required for GHR downregulation. Similarly, EGF-stimulated activation of Src kinase leads to tyrosine phosphorylation of clathrin, and as phosphorylation is required to recruit clathrin to the membrane, this observation strongly suggests a role for c-Src in EGF receptor endocytosis (34). This is not the case for the $GHR_{4P\text{-}A}$, since inhibition of JAK2 and consequently of Src does not affect GHR clathrin-dependent endocytosis. Furthermore, it has recently been published that neither activation of the erythropoietin receptor, another member of the cytokine family, nor JAK2 tyrosine kinase activity are required for internalization of bound erythropoietin (36). Taken together, these results suggest that, for signaling receptors of the cytokine receptor superfamily, endocytosis follows a pathway distinct from signaling receptors of the RTK family.

Acknowledgements

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References:

1. Kelly, PA, Ali, S., Rozakis, M., Goujon, L., Nagano, M., Pellegrini, I., Gould, D., Djiane, J, Edery, M, Finidori, J., and Postel-Vinay, MC. (1993) Recent Prog. Horm. Res. 48, 123-164
2. Finidori, J. (2000) Vitamins and Hormones 59, 71-97.
3. Carter-Su, C., and Smit, L. S. (1998) Recent Prog. Horm. Res. 53, 61-83.
4. VanderKuur, J. A., Wang, X., Zhang, L., Cambell, G. S., Allevato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1994) J. Biol. Chem. 269, 21709-21717.
5. Frank, S.J., Gilliland, G., Kraft, A.S., and Arnold, C.S. (1994) Endocrinology 135, 2228-39

6. Argetsinger, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N., and Carter-Su, C. (1993) Cell 74, 237-244.
7. Sotiropoulos, A., Perrot-Applanat, M., Dinerstein, H., Pallier, A., Postel-Vinay, M.C., Finidori, J., and Kelly, P.A. (1994) Endocrinology 135, 1292-1298.
8. Han, Y., Leaman, D. W., Watling, D., Rogers, N. C., Groner, B., Kerr, I. M., Wood, W. I., and Stark, G. R. (1996) J. Biol. Chem. 271, 5947-5952.
9. Thirone, A. C., Carvalho, C.R. O., and Saad, M. J. A. (1999) Endocrinol. 140, 55-62.
10. Smit, L. S., Meyer, D.J., Billestrup, N., Norstedt, G., Schwartz, J., and Carter-Su, C. (1996) Mol. Endocrinol. 10, 519-533.
11. Wang, Y.-D., and Wood, W. I. (1995) Mol. Endocrinol. 9, 303-311.
12. Billestrup, N., Bouchelouche, P., Allevato, G., Ilondo, M., and Nielsen, J. H. (1995) Proc. Natl. Acad. Sci. USA 92, 2725-2729.
13. Sotiropoulos, A., Moutoussamy, S., Renaudie, F., Clauss, M., Kayser, C., Gouilleux, F., Kelly, P. A., and Finidori, J. (1996) Mol. Endocrinol. 10, 998-1009.
14. Goujon, L., Allevato, G., Simonin, G., Paquereau, L., Le Cam, A., Clark, J., Nielsen, J. H., Djiane, J., Postel-Vinay, M.C., Edery, M., and Kelly, P.A. (1994) Proc. Natl. acad. Sci. USA 91, 957-961.
15. Allevato, G., Billestrup, N., Goujon, L., Galsgaard, E. D., Norstedt, G., Postel-Vinay, M.C., Kelly, P.A., and Nielsen, J.H. (1995) J. Biol. Chem. 270, 17210-17214.
16. Argetsinger, L. S., Norstedt, G., Billestrup, N., White, M. F., and Carter-Su, C. (1996) J. Biol. Chem. 271, 29415-29421.
17. Roupas, P., and Herington, A. C. (1988) Mol. Cell. Endocrinol. 57, 93-99.
18. Murphy, L. J., and Lazarus, L. (1984) Endocrinol. 115, 1625-1632.
19. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. 15, 3806-3812.
20. Govers, R., van Kerkhof, P., Schwartz, AL., and Strous, GJ. (1997) EMBO J. 16, 4851-58
21. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. 18, 28-36
22. van Kerkhof, P., Govers, G., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575-1580.
23. Lamaze, C., and Schmid, S. L. (1995) J. Cell Biol. 129, 47-54.
24. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) Can. J. Physiol. Pharmacol. 74, 1095-1110.
25. Freedman, N. J., and Lefkowitz, R. J. (1996) Recent Prog. Horm. Res. 51, 352-353
26. Chen. (1987) Nature (lond.) 328, 820-823.
27. Honegger, A.M. Dull, T.J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A.n and Schlessinger J. (1987) Cell 51, 199-209.

28. Alves dos Santos, C, van Kerkhof, P, and Strous, G. J. (2001) J. Biol. Chem. 276, 10839-46
29. Strous, G.J., van Kerkhof, P., Govers, R., Rotwein, P., and Schwartz, A.L. (1997) J. Biol. Chem. 272, 40-43.
30. Colosi, P., Wong, K., Leong, S.R., and Wood, W.I. (1993) J. Biol. Chem. 268, 12617-23.
31. Moller, C., Hansson, A., Enberg, B., Lobie, P. E., and Norstedt, G. (1992) J. Biol. Chem. 267, 23403-23408.
32. Moldrup, A., Billestrup, N., Dryberg, T., and Nielsen, J.H. (1991) J. Biol. Chem. 266, 17441-17445.
33. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996) J.Biol.Chem. 271, 13377-13384.
34. Wilde, A., Beattie, E. C., Lem, L., Riethof, D. A., Liu, S.-H., Mobley, W. C., Soriano, P., and Brodsky, F. M. (1999) Cell 96, 677-687.
35. VanderKuur, J.A., Butch, E.R., Waters, S.B., Pessin, J.E., Guan, K.L., and Carter-Su, C.(1997) Endocrinology 138, 4301-4307.
36. Beckman, D, Lin, L.L., Quinones, M.E., and Longmore, G.D. (1999) Blood 94, 2667-2675

Chapter IV

The Role of Oxidative Stress on the Turnover of the Growth Hormone Receptor

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Abstract

Oxidative stress activates the ubiquitin-proteasome system, and increases the cellular levels of ubiquitin-protein conjugates at the expense of free ubiquitin. In this report we demonstrate that growth hormone receptor (GHR) levels are reduced in cells subjected to oxidative stress. H_2O_2 treatment induced a decrease in the number of GHRs at the cell-surface, by both delaying its transport to the plasma membrane, and enhancing shedding activity. Both effects are independent of the UbE motif of the GHR as, upon stress, the endocytosis-deficient GHR-F327A behaves in a similar manner. These findings indicate that cells contain stress-induced mechanisms that regulate GHR availability at the cell-surface and diminish its biological activity, like protein synthesis and cellular growth, in order to enable the cell to recover from oxidative stress.

Introduction

The response of a cell to its external environment requires rapid and significant alteration of protein amount, localization and/or function. Several tightly regulated processes control synthesis, localization and degradation of such proteins. The ubiquitin-proteasome system is involved in intracellular proteolysis, cell cycle regulation, cellular growth and differentiation, receptor function, development and the stress response. Substrates regulated by this system include several cyclins, tumor suppressors, transcription factors, and cell-surface receptors. The target proteins are first tagged with ubiquitin molecules to form a polyubiquitin chain, which is specifically recognized by the multi-subunit proteasome complex, leading to their degradation. Attachment of ubiquitin was found to be important in defending against cellular damage caused by external influences including environmental insults, elevated temperatures, heavy metals, amino acid analogs, viral infection, oxidative and chemical damage, infections and mutations (1-4). In general, transcriptional induction of a set of genes whose products, known as stress proteins, enhances survival under stress conditions (for review: 5). Certain components of the ubiquitin pathway were found to be stress proteins, including ubiquitin itself and specific E2 enzymes, which expression is up-regulated in stressed cells (6). Imposition of stress is usually accompanied by a dramatic redistribution of ubiquitin, such that most of the ubiquitin in the cell becomes conjugated to substrate proteins (1, 7, 8). In yeast, the E2's UBC5 and UBC5 genes are important for the ubiquitination and turnover of normal short-lived proteins (6), but they are also involved in heat-shock and cadmium induced stress (2, 6). No E3 enzymes have yet been identified as stress proteins, nor has any E3 been shown to play an important role in the stress response. Protein synthesis inhibitors like cycloheximide, do not prevent stress-induced ubiquitin redistribution, suggesting that this event is mainly due to increased susceptibility of proteins to ubiquitination. However, up-regulation of ubiquitin and E2s might also contribute to increased conjugate levels (8).
Particularly, upon oxidative stress, both protein ubiquitination and proteasomal degradation have been demonstrated (8, 9). Oxidative stress damages cells, and has been implicated in several processes, including degenerative diseases of aging, Alzheimer's and Parkinson's disease, arthritis, atherosclerosis, and cancer (10-15). The exposure of cells to high levels of oxygen free radicals results in damage of proteins, lipids, DNA, and enzyme activity, affecting both cell proliferation and differentiation. Proteins containing free cysteine residues are targets for oxidants, where, for instance, phosphorylation/dephosphorylation activities are affected. Several proteins involved in signal transduction, including MAPKs, Raf-1, Ras, and growth factor receptors, such as the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), are phosphorylated and activated upon oxidative stress (16-20). For both the EGFR (21) and the chicken hepatic

lectin (CHL) receptor, that mediates endocytosis of glycoproteins terminating with N-acetlyglucosamine, ligand-dependent internalization was shown to become inhibited upon hydrogen peroxide (H_2O_2) stress (22). Possibly, H_2O_2 inhibits EGFR internalization by inhibition of Eps15 ubiquitination, known to be involved in the internalization process (21). It is thought that the increased EGFR phosphorylation upon H_2O_2 exposure, is a result of tyrosine phosphatase inactivation (19, 23). Following cellular exposure to oxidants, the levels of the cellular sulfhydryl reductant glutathione (GSH) decrease, while the levels of its oxidized form (GSSG) increase. Upon severe oxidative stress, ubiquitination of proteins is also decreased, suggesting that oxidative stress interferes with E1/E2 activities (24, 25). These enzymes contain active site sulfhydryls that might be covalently modified (thiolated) upon enhancement of GSSG levels (glutathiolation). Glutathiolation might regulate the levels of ubiquitinated proteins in response to oxidative stress (25).

Previously, we have shown that the ubiquitin system is required for ligand-induced growth hormone receptor (GHR) internalization (26). In the presence of ligand, GHR endocytoses rapidly via clathrin-coated pits (27), and its degradation occurs at least partially within the lysosome (28). In a chinese hamster cell line carrying a temperature-sensitive E1 enzyme (ts20 cells), inactivation of E1 results in accumulation of non-ubiquitinated GHRs at the plasma membrane, while internalization of the transferrin receptor is unaffected (26). Therefore, binding of GH induces ubiquitination, internalization and degradation of the GHR. A 10 amino acid motif, including Phe-327 within the GHR cytosolic tail (UbE motif), was found to be involved in both receptor ubiquitination and GH-induced receptor endocytosis (29). Mutation of Phe-327 leads to an inhibition of receptor ubiquitination and endocytosis. In addition to an active ubiquitin conjugation system, GHR internalization requires proteasomal action (30). Specific proteasomal inhibitors block GH uptake of the full-length GHR, while a truncated receptor can endocytose undisturbed. In addition, ubiquitination of the GHR itself does not seem to be important for internalization, as a truncated GHR with all its lysines mutated to alanines, was still able to internalize GH (31).

In this report, we addressed the role of oxidative stress on the turnover of the GHR. We show that upon H_2O_2 treatment, levels of ubiquitin-protein conjugates increased, while free ubiquitin decreased. As a consequence, GHR levels at the cell-surface decreased while GHR precursor forms in the cell remained unaffected, both by delaying its transport to the plasma membrane and inhibiting its downregulation from the cell-surface; in addition, the amount of growth hormone binding protein (GHBP) was increased. Furthermore, we observed that this effect was independent of the UbE motif of the GHR as, upon stress, the GHR-F327A behaviour is similar to the wtGHR. In absence of ligand, a small percentage of the GHRs at the cell-surface was ubiquitinated (29). However, stress and the consequent activation of the ubiquitin system did not increase the receptor's downregulation by triggering endo-

cytosis, but instead, influence the turnover of the receptor in the cell.

Experimental Procedures

Cells and Materials
Chinese hamster ts20 cells, containing a thermolabile ubiquitin-activating enzyme E1, were stably transfected as described previously (26). The internalization-deficient mutant GHR-F327A was constructed by site-directed mutagenesis, cloned and transfected into ts20 cells as described before (29). Stable, geneticin-resistant transfectants were grown in Eagle's minimal essential medium (MEM-α) supplemented with 10% fetal bovine serum (FCS), penicillin and streptomycin, 4.5 g/liter glucose, and 0.45 mg/ml geneticin. For experiments, cells were grown in the absence of geneticin at approximately 70% confluence.
Antibody to GHR was raised against amino acids 271-381 of the cytoplasmic tail (anti-GHR(T)) as previously described (26). Antibody (Mab5) recognizing the lumenal part of the GHR was from AGEN Inc., Parsippany, NJ. Antiserum against human GH was raised in rabbits. Antiserum specific for protein-ubiquitin conjugates was a generous gift from Dr. A. Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). The GH antagonist, B2036, was kindly supplied by Willem F. Bennett of Sensus Drug Development Corporation (Austin, TX). Culture medium, fetal calf serum (FCS), geneticin and hydrogen peroxide (H_2O_2) were purchased from Gibco. LipofectAMINE was from Life Technologies, Inc. Transferrin (Tf) was purshased from Sigma (St. Louis, MO).

Plasmids and transfection
Wild-type human GH cDNA was cloned into pcDNA3.1 vector (Invitrogen BV/Novex), and used for transiently transfections. Subconfluent cultures of ts20 cells were 30% confluent when transfected with 1 μg cDNA per dish, using LipofectAMINE according to the protocol. Cells were used for experiments 48 hours after transfection.

B2036 binding and internalization
^{125}I-labeled GH antagonist (^{125}I-B2036) was prepared by using chloramine T (19). Ts20 cells stably transfected with wtGHR, were grown in 12 well-plates, washed and incubated with MEMα supplemented with 20 mM Hepes (MEMα/Hepes), 10% FCS, and 4.5 g/liter glucose with or without 10 mM H_2O_2 for 30 min at 30^{0}C. A short incubation of 6 min with 8 nM ^{125}I-B2036 in MEMα/Hepes in the absence or presence of excess unlabeled GH, was followed by different periods of chase from 0 to 1 hour at 30^{0}C. At the indicated times, the medium was collected and precipitated with one volume of ice-cold 20% trichloroacetic acid (TCA) for 30 min on ice. Acid soluble and insoluble radioactivity were determined, after centrifugation, in

the supernatant and pellet, and used as a measurement for degraded or undegraded ligand, respectively. The cells were washed with ice-cold PBS, membrane-associated B2036 was removed by acid wash (0.15 M NaCl, 0.05 M glycine, pH 2.2), and internalized B2036 was determined by measuring the radioactivity after solubilization of the acid-treated cells by 1 M NaOH. The cell extracts were counted in a LKB gamma counter.

Transferrin internalization

Transferrin (Tf) (Sigma) was saturated with Fe3+ and labeled with ^{125}I (^{125}I-Tf) using iodo-beads (Pierce) according to standard procedures. Ts20 cells, grown in 6 well-plates, were first depleted from serum Tf by extensive washing and incubation with 20 mM MEMα/Hepes, 4.5 g/liter glucose and 0.1% bovine serum albumin (BSA) with or without 10 mM H_2O_2 for 30 min at 30^0C. 2μg/ml ^{125}I-Tf was added in each well in the absence or presence of excess unlabeled Tf (200 μg/ml), and followed for different periods of time at 30^0C. The cells were first extensively washed with MEMα/Hepes, containing 4.5 g/liter glucose and 0.1% BSA, and further washed with ice-cold PBS. Membrane-associated Tf was removed by acid wash (20 mM MES pH 5.0, 130 mM NaCl, 50 μM desferal, 2 mM $CaCl_2$, 0.1% BSA), followed by a wash with MEMα/HEPES pH 7.4. Internalized Tf was determined by measuring the radioactivity after solubilization of the acid-treated cells with MEMα/HEPES and 0.1% BSA containing 50 μM desferal at 30^0C. The cell extracts were counted in a LKB gamma counter.

Cell lysis and western blotting

Cells, grown in dishes in presence of MEM-α supplemented with 10% FCS, penicillin and streptomycin, 4.5 g/liter glucose, and 0.45 mg/ml geneticin, were treated with 10 mM H_2O_2 for different periods of time at 30^0C. Cells were lysed on ice in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were cleared by centrifugation. GHBP appearance in the medium was measured by collecting the medium of cells incubated with or without H_2O_2 for different periods of time. Both lysates and medium were analyzed by gel electrophoresis in the presence of SDS (SDS-PAGE, 7.5% or 4-20% polyacrylamide gradient gel) and transferred to polyvinylidene difluoride paper. The blots were immunostained with either GHR (Mab5), anti-GHR (T), or GH antibodies. After incubating the blots with rabbit anti-mouse IgG (RAMPO) or protein A conjugated to HRP, antigens were visualized through the ECL system (Boehringer Mannheim).

Metabolic labeling

For metabolic labeling, the cells were incubated in methionine-free MEM for 30 min and then [^{35}S]methionine (3.7 MBq/ml TRAN-^{35}S labelTM, 40 TBq/mmol,

ICN, Costa Mesa, CA) was added and labeled for 10 min at 30^{0}C in a CO_2-incubator. The radioactivity was chased for different times with or without 10 mM H_2O_2 in the presence of MEMα containing 10% FCS, 4.5 g/liter glucose, and 100 μM unlabeled methionine. The cells were further washed and lysed on ice in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were cleared by centrifugation. Immunoprecipitations were carried out with GHR(T) for 2 hours at 0^{0}C in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin (BSA), 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. Protein A-agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immuno-complexes. The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. The samples were boiled in DTT and analyzed by SDS-PAGE. The radioactivity was determined using a StormTM imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image QuaNT software version 4.2a.

GH Secretion

Ts20 cells transiently transfected with a pcDNA3-hGH construct were [^{35}S]methionine pulse-chased as described above. The radoactivity was chased for different times, with or without 10 mM H_2O_2, in presence of dialysed MEMα containing 10% FCS, 4.5 g/liter glucose, and 100 μM unlabeled methionine. The medium was collected at the time points indicated and cells lysed as described above. Both medium and cleared lysates were subjected to immunoprecipitation with GH antibody, and analyzed as above described.

Results

Oxidative stress decreases mature GHR levels independently of its UbE motif

GHR endocytosis is mediated by the ubiquitin-proteasome system, in particular, through the UbE motif (26, 31). Upon GH stimulation, the GHR is ubiquitinated and internalized, while in the mutated receptor (GHR-F327A), both ubiquitination and internalization are blocked. Since the ubiquitin system plays a key role in GHR downregulation, we asked whether GHR mature and precursor levels are affected by oxidative stress. A time course experiment was performed in which H_2O_2 was added to ts20 cell lines stably transfected with wtGHR (Fig. 1). Western blots of lysates blotted with receptor antibodies show that, during time, the levels of mature forms of the receptor are decreasing, in contrast to control cells (untreated cells), while the precursor forms are stabilized or even increased. To determine whether the UbE motif, and, possibly, the ubiquitin conjugation system are involved, the same experiment was performed for the cells expressing GHR-F327A. As seen in

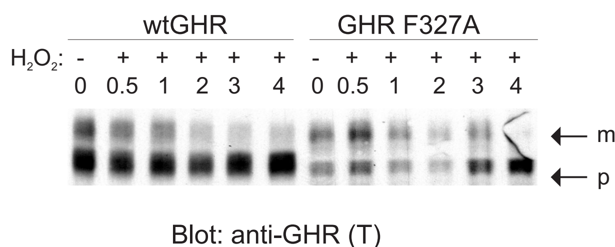

Fig. 1 **Cellular levels of the GHR are affected by H_2O_2 stress**
WtGHR and GHR F327A cells were incubated at 30°C without (control) or with 10 mM H_2O_2 for the time periods indicated. Cell lysates were prepared and subjected to immunoblot analysis using anti-GHR(T). Arrows indicate the *p*, precursor GHR (110 kDa), and the *m*, mature GHR (130 kDa).

Fig. 1, the GHR-F327A mutant behaved in a similar manner as the wtGHR upon H_2O_2 treatment. These results suggest that the H_2O_2-induced downregulation is independent of the UbE motif, and consequently of ubiquitination and endocytosis of the GHR.

GHR internalization is inhibited upon oxidative stress, while Tf receptor internalization is unaffected

Since steady state levels of GHR are affected by oxidative stress, we investigated whether H_2O_2-induced stress had an effect on regulating the amount of mature GHR at the plasma membrane (Fig. 2). H_2O_2 was added to the cells and internalization of ^{125}I-B2036, added during 6 minutes, was monitored for different periods of time, whereafter the amounts of intracellular and degraded ligand in the medium were determined. B2036 was used as a tag, since this ligand can bind a single receptor and prevent dimerization, mimicking the internalization of a non-stimulated receptor at the cell-surface. In both control and treated cells, the amount of cell-surface bound ^{125}I-B2036 decreased during time (Fig. 2A); incubation at 30^{0}C for 30 minutes resulted in uptake of about 30% of the antagonist in control cells, while only 10% was internalized in H_2O_2-treated cells (Fig. 2B). Thus, in cells treated with H_2O_2, the amount of intracellular B2036 was markedly decreased as compared to the control levels, and consistently, its degradation was also inhibited (Fig. 2C). The measurement of TCA soluble radiactivity showed that for control cells, internalized B2036 was rapidly degraded, starting after 20 minutes of chase, while in presence of H_2O_2, the degradation of B2036 was almost completely inhibited. As in H_2O_2-treated cells, B2036 binding sites at the plasma membrane decreased and its internalization was inhibited, we determined whether non-degradable products would appear in the medium during time. Indeed, Fig. 2D shows that in stress conditions, the appearance of TCA precipitable radioactivity in the medium was increased to 45% compared to 10% in control cells at 60 minutes. These results clearly show an effect of oxidative stress on GHR downregulation.
To examine whether H_2O_2 affected clathrin-mediated endocytosis in general, ^{125}I-Tf uptake was determined in cells treated with H_2O_2 (Fig. 3). A short time course was

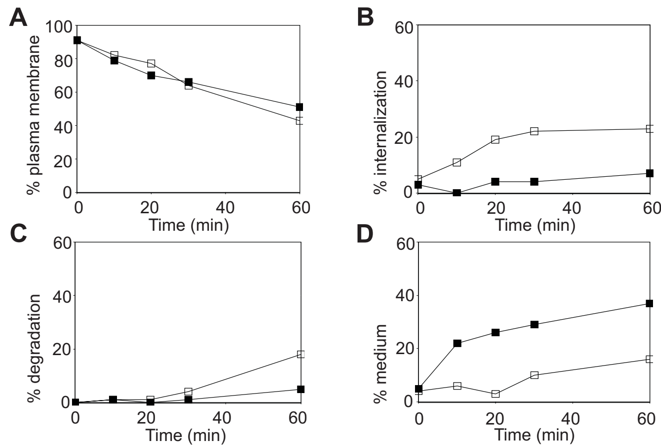

Fig. 2. **Internalization kinetics of wtGHR in H_2O_2 stress conditions.**
GH internalization was quantitated by [^{125}I]B2036 uptake. WtGHR cells in control (open symbols) and H_2O_2-treated (closed symbols) cells were incubated with 8 nM [^{125}I] B2036 in the absence or presence of excess unlabeled GH and chased for the indicated periods of time at 30^{0}C. **A**, specific cell-surface associated [^{125}I]B2036 and **B**, specific internalized [^{125}I]B2036, were expressed as the percentage of total radioactivity. TCA-soluble (**C**) and -insoluble (**D**) radioactivity in the incubation medium was determined and expressed as the percentage of total cell-associated and TCA-soluble label at 60 min.

performed to measure internalization, rather than recycling. As shown in Fig. 3, ^{125}I-Tf internalization was only slightly affected in cells treated with H_2O_2, when compared with control cells: only during the first 2 minutes H_2O_2 exerted an effect on Tf uptake. Thus,GHR downregulation is specifically affected by oxidative stress while endocytosis of other plasma membrane proteins is hardly affected.

GHR transport to the plasma membrane is delayed in the presence of H_2O_2

To further analyze the role of H_2O_2 on GHR regulation, we determined whether transport from the ER to the plasma membrane plays a role in the fast decrease of ligand binding sites at the cell-surface. A pulse-chase labeling experiment with

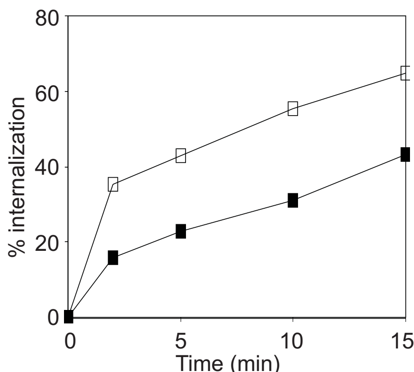

Fig. 3 **Transferrin internalization upon H_2O_2 stress treatment.**
Tf internalization was quantitated by [^{125}I]Tf uptake. WtGHR cells in control (open symbols) and H_2O_2-treated cells (closed symbols) were incubated with 8 nM [^{125}I]Tf in the absence or presence of excess unlabeled Tf and chased for the indicated periods of time at 30^{0}C. Specific internalized [^{125}I]Tf is expressed as the percentage of total radioactivity after short times of ligand uptake.

[35S]methionine was performed, followed by an immunoprecipitation of the GHR. As seen in Fig. 4A, the precursor form of the GHR rapidly disappeared during time, and was converted to the mature receptor within 1 hour of chase, after which rapid degradation occurred. When H_2O_2 was added, the precursor form of the GHR was stabilized, while mature GHR only became visible after 2 hours. Quantification of these results (Fig. 4B) indicates that in stressed cells, GHR maturation was at least two-fold slower. To investigate whether transport to the plasma membrane was affected by oxidative stress in general, GH secretion was monitored in a pulse-chase labeling experiment using transiently transfected ts20 cells. As seen in Fig. 5A, after 2 hours of chase, GH was detected in the medium in both control and H_2O_2-treated cells (lanes 4 and 7, respectively). Untransfected ts20 cells were used as a negative control (lane 14). Quantification of the results of Fig. 5B shows that, in both control and H_2O_2-treated cells, GH was normally secreted into the medium. Accordingly, the amount of labeled GH decreased in the cell in both situations, indicating that oxidative stress plays no significant role in normal protein secretion in these cells.

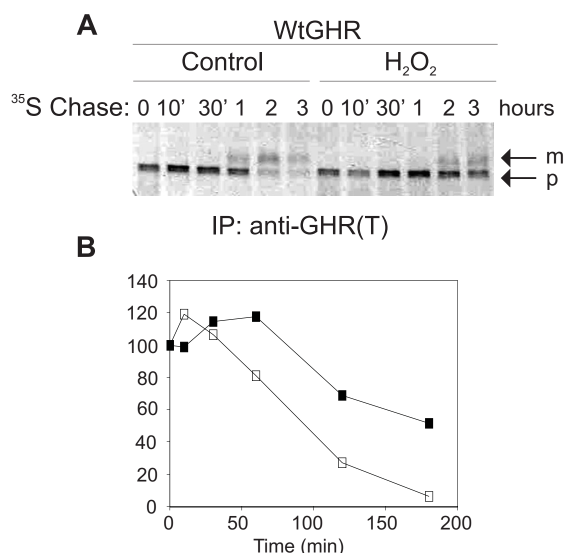

Fig. 4. **Pulse-chase labeling with [^{35}S] methionine of wtGHR upon H_2O_2 stress.**
WtGHR-expressing cells were pre-incubated with methionine-free MEMα for 30 min, labeled with [^{35}S]methionine for 10 min at 30^0C and chased in the absence of radioactivity in MEMα supplemented with 0.1% BSA, 100 µM methionine with or without 10mM H_2O_2 for the indicated times. **A**, cell lysates were immunoprecipitated with anti-GHR antibody (anti-GHR(T)).; *p*, precursor GHR (110 kDa); *m*, mature GHR (130 kDa). **B**, Radioactivity was quantified by using ImageQuant (Molecular Dynamics) and expressed as a percentage of the radioactivity incorporated in the precursor GHR after the pulse labeling during time. Precursor wtGHR is represented in absence of H_2O_2 by open symbols and in presence of H_2O_2 by closed symbols.

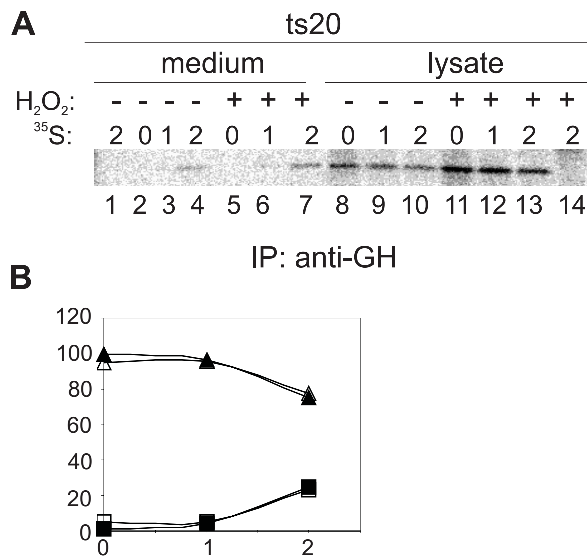

Fig. 5 **Pulse-chase labeling with [^{35}S]methionine of GH sorting upon H_2O_2 stress.**
GH-transiently transfected ts20 cells were pre-incubated with methionine-free MEMα for 30 min, labeled with [^{35}S]methionine for 10 min at 30^{0}C and chased in MEMα supplemented with 0.1% BSA, 100 µM methionine and with or without 10mM H_2O_2 for the indicated times. **A**, cell lysates were immunoprecipitated with anti-GH antibody (anti-GH). Untransfected ts20 cells were used as a negative control (lane 14); **B**, radioactivity was quantified by using ImageQuant (Molecular Dynamics) and expressed as the percentage of total radioactivity in both the cell and medium for each time point. GH appearance in the medium is represented in absence of H_2O_2 by open squares and in presence of H_2O_2 by closed squares. GH disappearance in the cells is represented in absence of H_2O_2 by open triangles and in presence of H_2O_2 by closed triangles.

Oxidative stress increases GHBP levels in the medium

Next, we addressed the question what happens to the GHR at the cell-surface in oxidative stress, in which internalization is inhibited and, at the same time, ligand binding sites decrease. Normally, the GHR is sensitive to proteolytic cleavage at the cell-surface, in particular by a metalloprotease called tumor necrosis factor (TNF)-α-converting enzyme (TACE or ADAM-17) (32), which generates a soluble circulating form of the receptor in the medium/blood. This process, also known as shedding, leads to the appearance of GH binding protein (GHBP) which is identical to the extracellular domain of the GHR and competes with the receptor for ligand-binding (33). To determine whether this process is affected in stressed cells, the appearance of GHBP in the medium was determined during time in wtGHR-transfected cells treated with H_2O_2 (Fig. 6A). Samples from the medium were collected at different times and analyzed by western blot with an antibody recognizing the extracellular domain of the GHR/GHBP. As seen in Fig. 6A for the wtGHR, H_2O_2

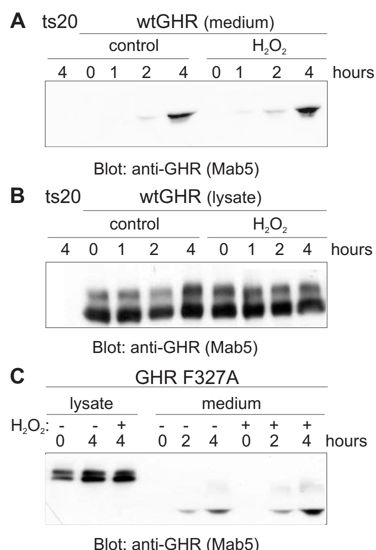

Fig. 6. **Effect of H_2O_2 on the shedding of the GHR.**
Cells expressing wtGHR and GHR-F327A were incubated without or with H_2O_2 for different periods of time at 30^0C. After each time point, the medium was collected and analyzed by Western blotting using an anti-GHR (Mab5) (**A**). As a control, the cells were lysed and subjected to the same procedure (**B**).

incubation lead to an increase of GHBP in the medium, when compared to untreated cells. In particular, after 2 hours, a difference between control and H_2O_2-treated cells is evident. Fig. 6B demonstrates that the appearance of GHBP in the medium during time is not due to an increased amount of cells in the experiment. To further determine the role of the UbE motif in this process, the same experiment was performed for the GHR-F327A cells. As seen in Fig. 6C, GHBP appearance in the medium was increased in H_2O_2-treated cells just as for the wtGHR cells, with similar amounts of receptor for each lane, reflecting again that the increased shedding is independent of the UbE motif. These results indicate that shedding is increased in stressed cells and it explains why less intact receptors are found at the cell-surface and, as shown in Fig. 2A, the amount of ligand binding sites are decreased during time.

Discussion

The ubiquitin-proteasome system is known to play a role in selective degradation of cytosolic and nuclear proteins (34) and to be involved in the degradation of misfolded endoplasmic reticulum proteins (35). Stress, viral infection, oxidative and chemical damage trigger vital cellular response, in particular by activating the ubiquitin-proteasome system, either to repair the damage, or to limit its toxicity to the cell. In response to several types of stress, organisms ranging from bacteria to humans induce several highly conserved gene families called heat shock proteins (Hsps). In yeast, the C-type cyclin Ume3p, part of a complex necessary for repression of genes of the Hsp70 family, is rapidly degraded by the ubiquitin-proteasome system in cells subjected to heat shock (36), suggesting that degradation of this pro-

tein is part of the cellular response to stress. In mammalian cells, the p53 tumor suppressor protein, which is normally degraded through the proteasome following interaction with the ubiquitin ligase Mdm2 (37, 38), becomes activated by diverse stress signals, resulting in stabilization and accumulation of p53 (39). In this way, p53 can function to inhibit tumor growth by activating target genes that mediate both cell cycle arrest and apoptosis in stressed cells.

In this study, we addressed the role of oxidative stress in modulating GHR levels in the cell and its effect on receptor availability. The GHR is normally downregulated by the ubiquitin-proteasome system upon ligand binding, and depends on ubiquitin conjugation for its endocytosis (26). The GHR, when at the cell-surface and in absence of ligand, is subjected to shedding, a process that requires the enzyme TACE, releasing GHBP due to the cleavage of the extracellular domain of the receptor (32). Upon ligand binding, the extracellular domain is protected from shedding (cleavage), and the GHR becomes ubiquitinated in clathrin coated pits (40), triggering clathrin-dependent endocytosis. Upon stress, the ubiquitin system is triggered to activate or downregulate specific proteins in the cell, and possibly also the GHR. Herein, we present evidence that in oxidative stress, the number of GHRs at the cell-surface is significantly reduced. H_2O_2 treatment induces a decrease in mature levels of the GHR in ts20 cells (Fig. 1), partially due to a delay in receptor maturation (Fig. 4). A delay of 30 minutes occurs in the maturation of the GHRs in the cell upon oxidative stress, and inhibition of the transport from the ER to Golgi leads to approximately 50% less receptors at the cell-surface at its steady state level (Fig.1). This stress-induced effect is specific for the GHR, as other secretory proteins, like GH itself, are not affected in their transport to the plasma membrane (Fig. 5). These results partially explain the decrease of mature GHR levels at the cell-surface. Furthermore, both wtGHR and the internalization mutant GHR-F327A show the same effect on protein turnover, suggesting that the UbE motif plays no significant role in the delayed transport to the cell-surface. This does not exclude the possibility that the downregulation of the GHR is controlled by the ubiquitin system, as another region/motif could be involved or even a protein attached to the receptor's tail could be somehow regulated by the ubiquitin system. By testing the effect of oxidative stress on the receptor levels at the plasma membrane, we observed that the ligand-binding sites at the cell-surface decrease faster in H_2O_2-treated cells than in control cells (Fig. 2A and results using GH, not shown). However, the decrease of GHR at the cell-surface was not due to a faster internalization from the cell-surface, as assayed by the GH-antagonist B2036 (Fig. 2B). Instead, the receptor remained longer at the cell-surface than in control cells, indicating that, other factor(s) are contributing to this downregulation of the GHR upon oxidative stress. This stress-induced inhibition is specific, as it is not observed in other plasma membrane proteins, like for instance, the Tf receptor which internalizes normally in H_2O_2-treated cells (Fig. 3). Furthermore, TCA-soluble products appear in the medi-

um in control cells after 20 minutes of B2036 uptake, while in H_2O_2-treated cells, the appearance of such products is significantly decreased (Fig. 2C). In contrast, TCA-insoluble products are increased several-fold in stressed cells (Fig. 2D), indicating that the decrease of ligand-binding sites at the cell-surface was due to the action of a protease that cuts the receptor from the extracellular domain. Indeed, an enhancement of the shedding activity in the GHR was observed in stressed cells (Fig. 6). TACE catalyzes the shedding of GHR from its extracellular domain and can be induced by phorbol ester stimulation (41). Possibly, TACE-mediated GHR proteolysis might be regulated by both oxidative stress and the ubiquitin system pathways, explaining the appearance of higher levels of GHBP in the medium. Alternatively, as oxidative stress induces an inhibition of receptor downregulation from the cell-surface, more receptors at the cell-surface are available for shedding, inducing therefore an increase of GHBP levels. This is unlikely because shedding of the endocytosis-mutant receptor GHR-F327A is increased in oxidative stress conditions.

These findings demonstrate for the first time that the availability of the GHR might be regulated by the oxidative-stress response pathway, and that GHR levels at the cell-surface are significantly reduced in response to oxidative stress. H_2O_2 acts at three stages: it delays maturation and/or transport of the GHR to the cell-surface, it inhibits GHR endocytosis from the cell-surface, and it increases shedding activity. These combined factors lead to an efficient decrease in GHR's availability in the cell and, in this way, diminish the capacity of the GHR to stimulate protein synthesis and cellular growth, in order to give the cell the chance to first recover from the oxidative stress.

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Abbreviations

GHR, growth hormone receptor; GH, growth hormone; EGF, epidermal growth factor; E1, ubiquitin-activating enzyme; MEM, minimal essential medium; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

References

1. Finley, D., Ozkaynak, E., and Varshavsky, A. (1987) Cell 48, 1035-1046

2. Jungmann, J., Reins, H.-A., Schobert, C., and Jentsch, S. (1993) Nature 361, 369-371
3. Treger, J. M., Heichman, K. A., and McEntee, K. (1998) Mol. Cell Biol. 8, 1132-1136
4. Cheng, L., Watt, R., and Piper, P. W. (1994) Mol. Gen. Genet. 243, 358-362
5. Pickart, C. M. (1999) Stress Proteins , 133-152
6. Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 543-550
7. Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A., and Marcus, M. (1988) J. Biol. Chem. 263, 15726-15731
8. Shang, F., Gong, X., and Taylor, A. (1997) J. Biol. Chem. 272, 23086-23093
9. Ramanathan, M., Hassanain, M., Levitt, M., Seth, A., Tolman, J. S., Fried, V. A., and Ingoglia, N. A. (1999) NeuroReport 10, 3797-3802
10. Crawford, D. W., and Blankenhorn, D. H. (1991) Atherosclerosis 89, 97-108
11. Witztum, J. L., and Steinberg, D. (1991) J. Clin. Invest. 88, 1785-1792
12. Ames, N. B., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. USA 90, 7915-7922
13. Jenner, P. (1994) Lancet 344, 796-798
14. Keller, J. N., Pang, Z., Geddes, J. W., Begley, J. G., Germeyer, A., Waeg, G., and Mattson, M. P. (1997) J. Neurochem. 69, 273-284
15. Sziraki, I., Mohanakumar, K. P., Rauhala, P., Kim, H. G., Yeh, K. J., and Chiueh, C. C. (1998) Neurosci. 85, 1101-1111
16. Rao, G. N. (1996) Oncogene 13, 713-719
17. Gonzalez-Rubio, M., Voit, S., Rodriguez-Puyol, D., Weber, M., and Marx, M. (1996) Kidney Int. 50, 164-173
18. Rosette, C., and Karin, M. (1996) Science 274, 1194-1197
19. Knebel, A., Rahmsdorf, H. J., Ullrich, A., and Herrlich, P. (1996) EMBO J. 15, 5314-5325
20. Abe, M. K., Kartha, S., Karpova, A. Y., Li, J., Liu, P. T., Kuo, W.-L., and Hershenson, M. B. (1998) Am. J. Respir. Cell Mol. Biol. 18, 562-569
21. De Wit, R., Capello, A., Boonstra, J., Verkleij, A. J., and Post, J. A. (2000) Free Rad. Biol. & Med. 28, 28-38
22. Chiacchia, K. B., and Drickamer, K. (1984) J. Biol. Chem. 259, 15440-15446
23. Lee, S.-R., Kwon, K.-S., Kim, S.-R., and Rhee, S. G. (1998) J. Biol. Chem. 273, 15366-15372
24. Shang, F., and Taylor, A. (1995) Biochem. J. 307, 297-303
25. Jahngen-Hodge, J., Obin, M. S., Gong, X., Shang, F., Nowell Jr., T. R., Gong, J., Abasi, H., Blumberg, J., and Taylor, A. (1997) J. Biol. Chem. 272, 28218-28226
26. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. 15, 3806-3812
27. Roupas, P., and Herington, A. C. (1988) Mol. Cell. Endocrinol. 57, 93-99

28. Murphy, L. J., and Lazarus, L. (1984) Endocrinol. 115, 1625-1632
29. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) EMBO J. 16, 4851-4858
30. van Kerkhof, P., Govers, G., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575-1580
31. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. 18, 28-36
32. Zhang, Y., Jiang, J., Black, R. A., Baumann, G., and Frank, S. J. (2000) Endocrinology 141, 4342-4348
33. Mannor, D. A., Winer, L. M., Shaw, M. A., and Baumann, G. (1991) J. Clin. Endocrinol. Metab. 73, 30-34
34. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425-479
35. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) Science 273, 1725-1728
36. Cooper, K. F., Mallory, M. J., Smith, J. S., and Strich, R. (1997) EMBO J. 16, 4665-4675
37. Haubt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Nature 387, 296-299
38. Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. (1997) Nature 387, 299-303
39. Ashcroft, M., Taya, Y., and Vousden, K. H. (2000) Mol. Cell. Biol. 20, 3224-3233
40. van Kerkhof, P., Sachse, M., Klumperman, J., and Strous, G. J. (2001) J. Biol. Chem. 276, 3778-3784
41. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) Nature 385, 729-733

Chapter V

Summarizing Discussion

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The signal transduction of the GHR is regulated by the ubiquitin-proteasome system

The results presented in chapter 2 indicate that GH-induced tyrosine phosphorylation of the GHR and JAK2 are prolonged in presence of proteasomal inhibitors, suggesting a role of the ubiquitin-proteasome system in signal regulation. In several cytokine receptors the JAK/STAT pathway is known to be downregulated by the proteasome. Upon treatment with specific proteasomal inhibitors, both interleukin-2 and -3 and interferon-γ receptor showed a prolonged JAK/STAT activation as well as other signaling molecules like the MAP kinases (1, 2). Furthermore, endocytosis plays no significant role in this proteasome-dependent downregulation of both JAK2 and GHR activities. Since signaling by the GHR-F327A endocytosis-deficient mutant still depends on the proteasome for its downregulation, our data indicate that proteasomal action on signal transduction occurs at the cell-surface .

Phosphatases or SOCS?

Our results with GHR 1-399 and GHR 1-369 indicate that partial deletion of the C-terminal GHR tail leads to a prolonged JAK2 phosphorylation presumably due to loss of a negative regulator binding site and its consequent activation. Tyrosine phosphatases, SHP-1 and SHP-2, and SOCS proteins, CIS and SOCS3, are involved in the dephosphorylation/inactivation of JAK proteins and subsequent termination of GH-induced cellular growth (3- 6). Upon ligand binding, SHP-2 and the various SOCS are known to interact with the region proximal to the C-terminal cytosolic tail of the GHR or with JAK2 itself. One explanation might be that the phosphatase activity is modulated by proteasome function, perhaps by degrading an inhibiting complex in a similar manner as it occurs for the inhibitor of the transcription factor NF-κB (7). In support of this model, SHP-1 degradation has been shown to be ubiquitin-dependent in mast cells (8), suggesting that the proteasome is involved in SHP-1 regulation. However, SHP-1 does not directly interact with the cytosolic tail of the GHR, rendering our results difficult to interpret. SHP-2, however, has been shown to interact directly with the tail of the GHR (residues 484-620) and to associate with JAK2 via SIRPα1 (9). SIRPα1 acts as a negative regulator of GH signaling by its ability to bind SHP-2 (9), and the proteasome could therefore play a role in SHP-2/SIRPα1 association and binding to JAK2. As SHP-2 is known to associate to other signaling molecules as IRS-1 (10) and p85-PI-3K (11), future studies will indicate whether the MG132 effect on these molecules is directly related to SHP-2 activity. Another explanation for our results comes from the action of the negative regulators of GHR signaling, the SOCS proteins. It is assumable that activated JAK kinases themselves are subject to proteasome-mediated degradation, possibly through the action of SOCS. Support for this comes from the recent identified and highly conserved C-terminal homology domain of the SOCS proteins, termed the SOCS box, which mediates interactions with elongins B and C, which

in turn may couple SOCS proteins and their substrates to the proteasomal protein degradation pathway (12). SOCS-3 binds to the GHR close to the transmembrane domain and might inhibit JAK2 signaling by interfering with JAK2 binding to the box-1 region; SOCS-1 acts at the level of JAK2 tyrosine kinase, as JAK2 tyrosine phosphorylation is strongly inhibited by SOCS-1 protein (13) and, in addition, interacts with the C-terminal residues of GHR, which may serve to correctly orientate SOCS-1 or perhaps increase its affinity for the receptor-kinase signaling complex (13); SOCS-2 and CIS, although to a lesser extent than SOCS-3 and with different kinetics (13), bind the C-terminal cytoplasmic domain of the GHR and, in particular, the docking sites for STAT5 (14). The inhibition by CIS or SOCS-2 of GHR-JAK2 signaling to STAT5 could result from direct competition between STAT5 and either CIS or SOCS-2 for tyrosine-phosphorylated binding sites on GHR. Alternatively, the inhibition of GH signaling by CIS or SOCS-2 could involve SOCS/CIS protein ubiquitination followed by degradation of the SOCS/CIS-bound GHR-JAK2 complex. How SOCS proteins inhibit JAK kinase activity is still not clear, but analogous to the family of F-box-containing proteins, SOCS box interaction with elongins B and C potentiates interaction with the proteasome complex. Interestingly, elongin B contains an ubiquitin-like (UBL) sequence at its N-terminus (15), suggesting a model for the action of the SOCS proteins (See Fig. 3, Chapter 1). This model suggests that either through direct interactions of the elongin B UBL domain with the proteasome or through associated Cullin-2-induced ubiquitination of substrates and subsequent proteasomal association, JAK2 and the associated SOCS proteins may be destroyed (12). This would explain why, in presence of MG132, degradation of SOCS proteins and its associated proteins like JAK2 would be prevented, and therefore induce sustained activation of JAK2 and, consequently of the GHR.

In favor of this model, is the finding that CIS and SOCS-1 are degraded in a proteasome-dependent manner (12, 16), and that SOCS-1 was shown to program VAV for ubiquitin-proteasome-mediated degradation by acting as a substrate-specific recognition component of a VCB-like ubiquitin ligase complex (16). Furthermore, CIS became ubiquitinated and its degradation was inhibited in presence of proteasome inhibitors upon EPOR stimulation (17). CIS was further shown to inhibit GHR-JAK2 signaling by two distinct mechanisms: (i) a partial inhibition that is decreased at elevated STAT5b levels and may involve competition between CIS and STAT5b for common GHR cytoplasmic tail phosphotyrosine-binding sites, (ii) and by a time-dependent inhibition, not seen with SOCS-3, that involves proteasome action (18). GH was shown to induce degradation of CIS, but not SOCS-3, and the proteasome inhibitor MG132 blocked CIS degradation as well as its inhibitory action on STAT5b signaling (18). Proteasome-dependent degradation of CIS, most likely in a (GHR-JAK2)-CIS complex, is therefore proposed to be an important step in the time-dependent CIS inhibition mechanism. Recently, it was demonstrated

that suppression of JAK2 activity was dependent on SOCS-1, where the SOCS box was suggested to mediate proteasomal degradation of JAK2 rather than JAK2 kinase inhibition (19). The SOCS box of SOCS-1 was found to interact with the E3 Cullin-2 and promoted ubiquitination of JAK2. This interaction demonstrated the substrate-specific E3 ubiquitin-ligase-like activity of SOCS-1 for activated JAK2, providing a novel strategy for the suppression of oncogenic tyrosine kinases (19).
Thus, the mechanisms for the termination of the GHR phosphorylation might depend on proteolysis: the regulation of both the phosphatases and the SOCS proteins activity. Further studies will be necessary to determine which of these groups of proteins, or a combination of both, is indeed involved in proteasome-dependent signal downregulation.

Signaling of the GHR continues in endosomes

Our data show that initiation as well as termination of both JAK2 and GHR phosphorylation can occur at the cell-surface. The observation that JAK2 phosphorylation was prolonged in the truncated forms of the GHR raised the question of what happens to the signal transduction of the GHR after its internalization. If the GHR is partially truncated by the proteasome during internalization, a truncated form might have a higher signaling capacity than the full-length GHR, once it has lost the binding sites for SHPs or SOCS. This implicates that JAK2 might still be active in endosomes. Co-immunoprecipitation of GH-GHR complexes containing either wtGHR or GHR 1-369 truncated form, before and after endocytosis showed that JAK2 as well as other activated proteins are bound to the GHR not only at the cell-surface but also intracellularly, suggesting that the GHR signal transduction continues in endosomes. This is not unexpected because GH keeps the two GHRs complexed after endocytosis. In this configuration, JAK2 has high affinity for the complex and will either rebind (if it was removed during passage of the coated pits) or will keep its position on the dimerized tails once internalized. The relevance for signal transduction in endosomes is not clear. It is possible that the signaling GHR-complexes in endosomes differ from those at the cell-surface. However, our results indicate that phosphorylated proteins in total and endocytosed GH complexes in wtGHR and GHR 1-369 look very similar, suggesting that GHR signal transduction continues or resumes after endocytosis.
Additionally, these results provide evidence that GHR is present in endosomes both in its full-length and truncated form, suggesting that the receptor might be partially downregulated by the proteasome. The GHR is C-terminally truncated already at the cell-surface as the GHR-F327A presented similar truncated forms.The truncated GHR can endocytose, complexed to GH, and even in presence of proteasome inhibitors its formation can still occur. It remains to be determined, whether this truncated GHR plays a role in signal transduction. Data from GHR truncated forms did present prolonged JAK2 phosphorylation during time, suggesting that such a

truncated receptor could also have an increased capacity of signaling.

Internalization and ubiquitination of the GHR are independent of GHR signal transduction via JAK2

In this study, as discussed in chapter 3, we examined the role of JAK2-mediated signal transduction in GHR internalization and downregulation. JAK2 is essential for the signaling of the GHR, as it is the first step in the activation of several cascades that lead to the biological responses of the GHR. Mutation of the attachment site for JAK2, box-1, in the GHR cytoplasmic tail resulted in the abrogation of JAK2 association with the GHR cytoplasmic domain, and in the complete absence of GHR and JAK2 phosphorylation. Although mutation of the box-1 region in the GHR did not affect the ability of the altered receptor to bind GH at the cell surface, it dramatically affected the receptor's capacity to couple GH binding to JAK2 activation and consequently of other possible signaling molecules. A previous report suggested that mutations in box-1 may lead to a block of GHR endocytosis (20). In our study, the same modification did not alter the rate and extent of receptor-bound GH internalization as compared to a functional GHR, nor did it change its maturation and transport to the plasma membrane. GH is still normally targeted for degradation in $GHR_{4P\text{-}A}$, indicating that intracellular sorting and transport to the lysosome is not affected in box-1-defective receptors. However, upon GH stimulation, wtGHR mature form showed relatively faster degradation, while addition of GH had no such clear effect on the turnover of the $GHR_{4P\text{-}A}$. This suggests that GH-dependent wtGHR accelerated degradation is mediated through a JAK2-dependent process that is absent in $GHR_{4P\text{-}A}$, indicating that signal transduction is contributing to a faster endocytosis/degradation of the GHR. This might be due to the fact that the signal transduction pathway via STAT or MAP kinase stimulates the clathrin-mediated endocytosis, a phenomenon known from the EGF receptor studies (21).
In addition, both wtGHR and mutated receptor were still normally ubiquitinated and remained dependent on both an intact ubiquitin system and on proteasomal action for its GH-dependent internalization. Thus, GHR/JAK2 signal transduction plays no role in the binding of the E2/E3 enzymes, members of the ubiquitin conjugation system, to the receptor. Furthermore, the motif at the GHR tail responsible for the proteasome action (22) does not interact with signaling proteins nor is such an interaction or phosphorylation itself important for the proteasome action. Together, these results demonstrate that the molecular mechanism which underlies the ubiquitin-proteasome system-dependent endocytosis of the GHR does not require the presence nor the activity of JAK2 and its signaling molecules.
Thus, the ubiquitin-proteasome system independently regulates the signal transduction capacity of the GHR in two ways: first, it determines the rate of endocytosis via the UbE-motif in the GHR tail, and second, it determines the signaling time via JAK2 and SOCS. Both mechanisms appear to be independently regulated by the

same system, probably via completely different E2/E3 enzyme systems.
In addition to the dispensability of JAK2 activity and receptor tyrosine phosphorylation, recruitment of Grb2 to bind the receptor via SHC and consequent activation of the MAPK pathway is also not required for GHR downregulation (23). Cells expressing truncated receptors that lack this domain and are therefore unable to associate with Grb2, can internalize the truncated receptor as efficiently as cells containing wtGHR. Similarly, Src, known to be essential for phosphorylation of clathrin and consequent EGF receptor endocytosis (24), does not play any role in GHR clathrin-dependent endocytosis. Furthermore, it has recently been published that neither activation of another member of the cytokine family, the erythropoietin receptor, nor JAK2 tyrosine kinase activity are required for internalization of bound erythropoietin (25). Taken together, these results suggest that, for signaling receptors of the cytokine receptor superfamily, endocytosis follows a pathway distinct from signaling receptors of the RTK family.
Thus, GHR ubiquitination, endocytosis and degradation occur independently of GHR signal transduction via JAK2. We conclude that, while endocytosis and degradation require the ubiquitin system, they are independent of GHR signal transduction.

GHR availability at the plasma membrane is regulated by oxidative stress

In Chapter 4, we address the role of oxidative stress in modulating GHR levels in the cell and its affect on receptor availability. Oxidative stress activates the ubiquitin-proteasome system, and induces higher levels of ubiquitin-protein conjugates in the cells with concomitant lower free ubiquitin levels (26-29), triggering the activation or downregulation of specific proteins in the cell, and possibly also the GHR. Our data indicate that an immediate effect of oxidative stress, is a higher turnover rate of the GHR, and as a consequence the GHR levels are significantly reduced from the cell-surface. H_2O_2-induced decrease of GHR levels at the cell-surface is accomplished by: (i) delaying its transport to the plasma membrane, (ii) inhibiting its internalization and (iii) enhancing shedding activity. This stress-induced inhibition is quite specific, as it is not observed for other plasma membrane proteins, like the Tf receptor that can still internalize in H_2O_2-treated cells. In addition, this effect is independent of the UbE motif of the GHR, as, upon stress, the GHR-F327A behaves in a similar manner as the wtGHR. This does not exclude the possibility that the downregulation of the GHR is being controlled by the ubiquitin system, as another region of the receptor could be involved or even a protein attached to the receptor's tail could be somehow regulated by the ubiquitin system. The partial inhibition of GHR's transport to the cell-surface in H_2O_2-treated cells is specific for the GHR, as other secretory proteins, like GH itself, are not affected in their transport to the plasma membrane. The enhancement of the shedding activity in the GHR is

observed in stressed cells, and is probably due to the fact that TACE-mediated GHR proteolysis is up-regulated by both oxidative stress and the ubiquitin system pathways. Alternatively, as oxidative stress induces an inhibition of receptor downregulation from the cell-surface, more receptors at the cell-surface are available for shedding, inducing an increase of GHBP levels. Whether the two mechanisms are connected, is still unclear.
These findings demonstrate, for the first time, that the availability of the GHR might be regulated by the oxidative-stress response pathway, and, in this manner, diminish possible GHR biological effects, like protein synthesis and cellular growth, in order to give the cell the chance to first recover from oxidative stress.

"It is better to be approximately right
than to be precisely wrong".
Maynard Keynes

References:

1. Yu, C. L., and Burakoff, S. J. (1997) J. Biol. Chem. 272, 14017-20
2. Callus, B. A., and Mathey-Prevot, B. (1998) Blood 91, 3182-3192
3. Adams, T. E., Hansen, J. A., Starr, R., Nicola, N. A., Hilton, D. J., and Billestrup, N. (1998) J. Biol. Chem. 273, 1285-1287
4. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) Cell 80, 729-738
5. Hackett, R. H., Wang, Y. D., Sweitzer, S., Feldman, G., Wood, W. I., and Larner, A. C. (1997) J. Biol. Chem. 272, 111280-11132
6. Jiao, H., Berrada, K., Yang, W., Tabrizi, M., Platanias, L. C., and Yi, T. (1996) Mol. Cell. Biol. 16, 6985-6992
7. Yaron, A., Gonen, H., Alkalay, I., Hatzubai, A., Jung, S., Beyth, S., Mercurio, F., Manning, A. M., Ciechanover, A., and Ben-Neriah, Y. (1997) EMBO J. 16, 6486-6494
8. Piao, X., Paulson, R., van der Geer, P., Pawson, T., and Bernstein, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14665-14669
9. Stofega, M. R., Argetsinger, L. S., Wang, H., Ullrich, A., and Carter-Su, C. (2000) J. Biol. Chem.,
10. Myers, J., M. G., Mendez, R., Shi, P., Pierce, J. H., Rhoads, R., and White, M. F. (1998) J. Biol. Chem. 273, 26908-26914
11. Gesbert, F., Guenzi, C., and Bertoglio, J. (1998) J. Biol. Chem. 273, 18273-18281
12. Zhang, J. G., Farley, A., Nicholson, S. E., Willson, T. A., Zugaro, L. M., Simpson, R. J., Moritz, R. L., Cary, D., Richardson, R., Hausmann, G., Kile, B. J., Kent, S. B. H., Alexander, W. S., Metcalf, D., Hilton, D. J., Nicola, N. A., and Baca, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2071-2076
13. Ram, P. A., and Waxman, D. J. (1999) J. Biol. Chem. 274, 35553-25561
14. Hansen, J. A., Hansen, L. H., Wang, X., Kopchick, J. J., Gouilleux, F., Groner, B., Nielsen, J. H., Moldrup, A., Galsgaard, E. D., and Billestrup, N. (1997) J. Mol. Endocrinol. 18, 213-221
15. Lonergan, K. M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R. C., Conaway, J. W., and Kaelin, W. G., Jr. (1998) Mol. Cell. Biol. 18, 732-741
16. De Sepulveda, P., Ilangumaran, S., and Rottapel, R. (2000) J. Biol. Chem. 275, 14005-14008
17. Verdier, F., Chretien, S., Muller, O., Varlet, P., Yoshimura, A., Gisselbrecht, S., Lacombe, C., and Mayeux, P. (1998) J. Biol. Chem. 273, 28185-28190
18. Ram, P. A., and Waxman, D. J. (2000) J. Biol. Chem. 275, 39487-39496
19. Kamizono, S., Hanada, T., Yasukawa, H., Minoguchi, S., Kato, R., Minoguchi, M., Hattori, K., Morita, S., Kitamura, T., Kato, H., Nakayama Ki, K., and Yoshimura, A. (2001) J. Biol. Chem. Papers in Press

20. Allevato, G., Billestrup, N., Goujon, L., Galsgaard, E. D., Norstedt, G., Postel-Vinay, M. C., Kelly, P. A., and Nielsen, J. H. (1995) J. Biol. Chem. 270, 17210-17214
21. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996) J. Biol. Chem. 271, 13377-13384
22. van Kerkhof, P., Govers, G., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575-1580
23. Vanderkuur, J. A., Butch, E. R., Waters, S. B., Pessin, J. E., Guan, K. L., and Carter-Su, C. (1997) Endocrinol. 138, 4301-4307
24. Wilde, A., Beattie, E. C., Lem, L., Riethof, D. A., Liu, S.-H., Mobley, W. C., Soriano, P., and Brodsky, F. M. (1999) Cell 96, 677-687
25. Beckman, D., Lin, L. L., Quinones, M. E., and Longmore, G. D. (1999) Blood 94, 2667-2675
26. Finley, D., Ozkaynak, E., and Varshavsky, A. (1987) Cell 48, 1035-1046
27. Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A., and Marcus, M. (1988) J. Biol. Chem. 263, 15726-15731
28. Carlson, N., and Rechsteiner, M. (1987) J. Cell Biol. 104, 547-555
29. Shang, F., Gong, X., and Taylor, A. (1997) J. Biol. Chem. 272, 23086-23093