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Ubiquitin System-Dependent Regulation of Growth Hormone Receptor Signal Transduction

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Abstract The growth hormone (GH) receptor is a key regulator of cellular metabolism. Unlike most growth factor receptors, its downregulation is not initiated by its ligand. Like many growth factor receptors, specific molecular mechanisms guarantee that a receptor can signal only once in its lifetime. Three features render the GH receptor unique: (a) an active ubiquitination system is required for both uptake (endocytosis) and degradation in the lysosomes; (b) uptake of the receptor is a continuous process, *independent* of both GH binding and Jak2 signal transduction; (c) only the cell surface expression of *dimerised* GH receptors is controlled by the ubiquitin system. This system enables two independent regulatory mechanisms for the endocrinology of the GH/GHR axis: the pulsatile secretion of GH by the pituitary and the GH sensitivity of individual cells of the body by the effects of the ubiquitin system on GH receptor availability.

Abbreviations

AP2 Heterotetrameric adaptor protein complex 2
CIS Cytokine-inducible SH2 domain-containing protein
EGF Epidermal growth factor
EpoR Erythropoietin receptor
ER Endoplasmic reticulum
GH Growth hormone
GHR Growth hormone receptor
IGF Insulin-like growth factor
IRS Insulin receptor substrate
LDL Low-density lipoprotein
LRP LDL receptor-related protein
NGF Nerve growth factor
PDGF Platelet-derived growth factor
RTK Receptor tyrosine kinase
SE Sorting endosome
SH2 Src homology 2
SOCS Suppressor of cytokine signalling
STAT Signal transducer and activator of transcription
TfR Transferrin receptor
TMD Transmembrane domain

1 Introduction

For a long time, the predominant view on the effectiveness of growth factor receptors was linear: Gene expression regulation determines the number of growth factor receptors at the cell surface, whereas their ligands, the growth factors, delimit timing and intensity of signal transduction. A regulatory potential of receptor control and involvement of intracellular compartments was hardly appreciated. Although gene expression levels (mRNA concentrations) are principally important, recent discoveries reveal signalling scenarios which implicate the full extent of the vacuolar system and the surprising picture emerges that, once translated, receptors are regulated individually. For some receptors protein folding, quality control, oligomerisation and protein complex formation in the endoplasmic reticulum (ER) determine their cell surface expression; for others, ligand-induced endocytosis is a major signal transduction regulator. Selective molecular mechanisms at endosomes appear to be another important determinant in signalling capacity: They can either

send the receptors back to the cell surface for prolonged signalling or route them towards the lysosomes for destruction. In addition, specific locations can affect the signalling initiated at the cell surface, e.g. by attenuating, boosting or changing signalling pathways. Growth factor receptor signalling is not linear: Signals generated by one receptor can affect and activate other receptors both at the cell surface and in endosomes. In other scenarios proteolytic events convert portions of receptors into factors involved in DNA transcription. For the growth hormone receptor (GHR), cell surface expression and signalling modes are not less complex. The receptor has an extremely rapid turnover, dictated by the activity of the ubiquitin system, not by its ligand, the growth hormone (GH). All organelles of the vacuolar system are involved in its life and death, rendering it a textbook example.

This review discusses recent studies which have uncovered different determinants in the signalling of the GHR. The major focus is on the involvement of the ubiquitin conjugation system in the fate of the receptor. From various studies in yeast, consensus emerges that nutrient-regulated permeases depend on ubiquitination by the ubiquitin ligase Rsp5p for their degradation. In mammalian cells, several activated receptor tyrosine kinases (RTKs), such for epidermal growth factor (EGF), the hepatocyte growth factor, and platelet-derived growth factor (PDGF), depend on the ubiquitin ligase c-Cbl for their lysosomal degradation. In fact, the GHR is the only well-documented growth factor receptor whose removal from the cell surface depends on an active ubiquitination machinery. Unique in this event is that it is ligand independent. As for the RTKs, an equally important sorting step in controlling the signalling potential occurs at the level of the sorting endosomes (SEs). The GHR and its ligand GH are of basic importance for regulation of metabolism in humans, and therefore its cell biology must be carefully and precisely regulated.

2
GHR Traffic Control

2.1
The GHR

GH regulates postnatal growth as well as lipid and carbohydrate metabolism (Isaksson et al. 1985). Hyposecretion results in dwarfism, whereas hypersecretion leads to gigantism, a clinical condition known as acro-

megaly. The secretion of GH is regulated by a complex neuroendocrine system which involves both neural and feedback regulatory components. At least two hypothalamic hormones, a stimulatory GH-releasing hormone and an inhibitory hormone, somatostatin, generate a striking pulsatile pattern of GH release (Miller et al. 1982). Experimental animal studies have established that both growth and metabolic actions depend on the pattern of GH exposure, indicating that signalling is concentration- and time dependent (Jansson et al. 1982). Influences of gender, body composition and exercise play important roles in influencing circulating GH concentrations; secretion declines during normal aging, and many age-related changes, including osteoporosis and muscle atrophy, may be due, in part, to the decreased actions of GH and insulin-like growth factor 1 (IGF-1) (Casanueva 1992).

GH effects are mediated via the GHR, a member of the class I cytokine receptor superfamily which in addition includes the receptors for erythropoietin (Epo), prolactin, thrombopoietin, leptin, ciliary neurotropic factor, leukaemia inhibitory factor, granulocyte colony-stimulating factor and several of the interleukins. Although the overall homology between the members is low, some conserved motifs have been identified (reviewed in Bazan 1990). Their extracellular domain contains two or three pairs of disulfide-linked cysteine residues and a WSXWS (Trp, Ser, any amino acid, Trp, Ser) motif, which is indirectly involved in ligand binding (Carter-Su et al. 1996). The structure of the GHR is depicted in Fig. 1.

Strong proof for the functional significance of the GHR came from the demonstration of splice defects in the extracellular region of the GHR in patients with Laron syndrome (Godowski et al. 1989). This form of dwarfism, which is characterised by inherited GH insensitivity syndrome, was identified by Laron et al. and has a clinical phenotype of severe growth retardation with high circulating GH accompanied by low serum IGF-1 and IGF-binding protein-3, with no responsiveness to exogenous GH (Laron et al. 1966). In the inherited GH insensitivity syndrome, over 30 mutations in the GHR have been described, with the majority of the mutations in the exons which code for the extracellular domain of the receptor, interfering with GH binding (Rosenbloom 2000; Ross 1999). Recently, a heterozygous point mutation in the splice acceptor site, upstream of exon 9, was described. This mutation resulted in exon 9 being omitted from the GHR mRNA, creating a truncated receptor GHR(1–277) with only seven residues in the intracellular domain (Ayling et al. 1999; Iida et al. 1999). This receptor does bind GH but is unable to transmit the signal to the downstream signalling pathway. Al-

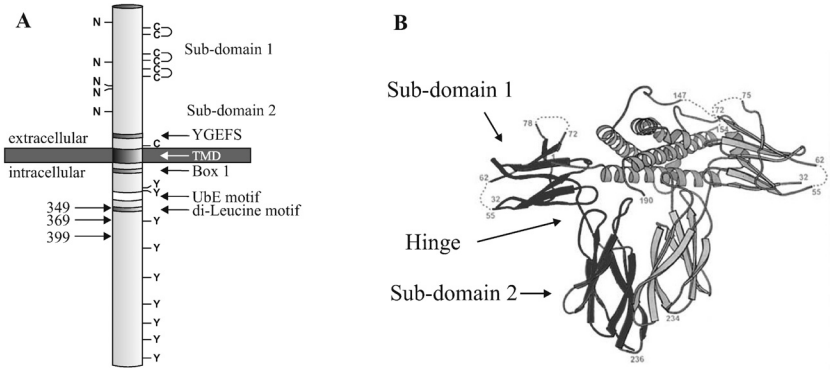

Fig. 1. A Schematic representation of the GHR. The GHR is a type I membrane protein (620 amino acid residues) and consists of an extracellular domain (246 aa), a single transmembrane domain (24 aa, *TMD*) and a cytoplasmic region (350 aa). The extracellular domain contains potential N-linked glycosylation sites (*N*), cysteine residues (*C*) and a WSxWS motif (in rabbit GHR: YGEFS) at the indicated positions. In the cytoplasmic domain, box 1, a proline-rich region required for signalling, and the ubiquitin-dependent endocytosis (*UbE*) and the di-leucine motifs are shown. Furthermore, rabbit GHR contains nine intracellular tyrosine residues, which are possibly involved in signalling. *Numbers to the left* indicate truncation mutants that are discussed in the text. **B** Structure of the GH-(GHR)2 complex. Ribbon rendering of the complex of one GH molecule and two GHR extracellular domains (shown in *dark and light grey*) (de Vos et al. 1992). The four α-helical bundle GH is seen at the *top* of the complex. The GHR extracellular domain is composed of two fibronectin type III domains (sub-domains 1 and 2), each consisting of seven β-strands. A four-amino acid hinge region separates the two sub-domains. (Adapted from de Vos et al. 1992)

ternative splices of the GHR, resulting in truncated isoforms, were identified in a permanent cell line of IM-9 cells and in a number of human tissues (Dastot et al. 1996, Ross et al. 1997). The alternative splices represented less than 10% of the total transcripts and are believed to act as dominant-negative inhibitors of GHR signalling by heterodimerisation with the full-length wild-type receptor [GHR(1–620)]. These findings suggest that differential expression of GHR isoforms could play a significant role in GHR signalling.

All cytokine receptors lack intrinsic kinase activity. Instead, they contain a cytosolic proline-rich domain, box 1, which functions as a binding site for members of the family of Jak2 tyrosine kinases. In the case of GHR, ligand binding leads to tyrosine phosphorylation of Jak2, the

receptor and downstream signalling molecules (reviewed in Argetsinger et al. 1993).

2.2 GHR Dimerisation and Traffic Control in the ER

On the basis of crystallographic data, it was postulated that a single GH molecule dimerises two GHR molecules through interactions between the membrane proximal extracellular regions of adjacent GHR molecules, referred to as sub-domain 2 (de Vos et al. 1992) (Fig. 1B). Mutations of conserved amino acids in this domain disrupt ligand-induced signal transduction, presumably by conformational changes rather than by preventing receptor dimerisation (Gent et al. 2002, Chen et al. 1997). However, dimerisation itself is not sufficient for signal transduction because administration of monoclonal antibodies directed against the extracellular domain of the GHR resulted in dimerised GHRs but failed to induce signal transduction. The GHR extracellular domain contains two sub-domains, which are separated by a hinge region (Fig. 1B) (de Vos et al. 1992). Mellado et al. who developed a monoclonal antibody against the extracellular hinge region of the GHR (Mellado et al. 1997), showed that antibody binding to the cell surface receptor increased upon GH binding, but not when the GH antagonist GH (G120R), mutated in the second GHR binding domain, was used. This suggests that signal transduction requires a specific orientation of two GHR molecules. In recent years, evidence has accumulated that GHR already exists as a preformed dimer at the cell surface. Cross-linking studies with ^{125}I-GH or GH antagonist revealed complexes similar in size which correspond to a GH-$(GHR)_2$ complex (Harding et al. 1996, van Kerkhof et al. 2002). Conclusive evidence was provided by the finding that immunoprecipitation of a full-length GHR resulted in co-immunoprecipitation of a truncated receptor which was not recognised by the antibody used in the immunoprecipitation (Gent et al. 2002). This interaction most likely reflects dimerisation, as larger complexes, containing more than two GHR molecules, have not been observed. Strikingly, dimerisation was not only observed between mature, glycosylated forms of the receptor but also between precursor species, which reside in the ER. These findings indicate that receptor dimerisation already occurs in the ER and is independent of ligand binding. The molecular mechanism of GHR dimerisation in the ER is still unknown, but there are indications that neither the transmembrane domain (TMD) nor the cytosolic domain of the receptor is involved in dimerisation. Elimination of 97% of the cytoplasmic tail of

the GHR does not effect the heterodimerisation of the truncated GHR with full-length GHR (Ross et al. 1997). Furthermore, mutating single and multiple amino acids of the TMD to alanine or replacement of the GHR TMD by a heterologous TMD does not disrupt GHR dimerisation (Gent and Strous, unpublished data), thereby rendering the extracellular domain an important player in receptor dimerisation. A role for the extracellular domain in the dimerisation process was suggested because replacement of the entire extracellular domain with part of the LDL receptor-related protein (LRP) results in monomeric chimers (Gent et al. 2003, Gent et al. 2002). On the other hand, the extracellular domain is not required to maintain the GHR in the dimerised state, because, once dimerised, protease digestion of the cell surface-localised GHRs does not disrupt dimerisation of the membrane-bound remnant protein. Therefore, the extracellular domain might be involved in the initial dimerisation process in the ER, whereas the TMD could be sufficient to maintain the GHR dimerised. A model of GHR dimerisation is shown in Fig. 2.

Although GH is not required for dimerisation , GH binding is essential for signal transduction. The activation of GHRs upon GH binding probably results in a conformational change of the receptor complex, leading to signal transduction events inside the cell. Mutations of amino acids of sub-domain 2 prevent the reorganisation induced by GH and result in signalling-deficient mutants without interfering with GHR

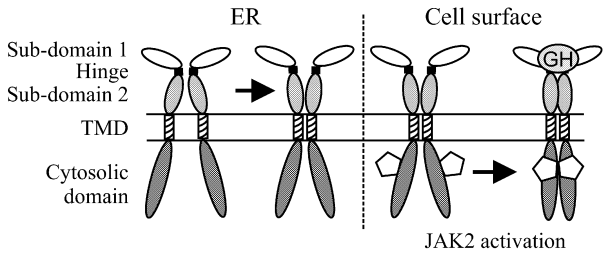

Fig. 2. GHR dimerisation in the ER and GH-induced activation at the cell surface. Dimerisation of GHRs occurs in the ER and is maintained during transport to the cell surface. The GHR extracellular domain is composed of an N-terminal GH binding sub-domain 1 and the membrane-proximal sub-domain 2, which can interact with sub-domain 2 of an adjacent GHR. ER chaperones facilitate this dimerisation. A hinge region separates the two domains. The TMDs of the dimerized GHRs are likely to be involved in stabilisation of the dimers. At the cell surface, GH binding induces a conformational change in the GHR-dimer, resulting in recruitment and cross-activation of two Jak2 tyrosine kinase molecules

dimerisation (Gent et al. 2003). These results link sub-domain 2 to signal transduction rather than dimerisation (Fig. 2). For various membrane proteins ligand-independent oligomerisation has also been reported. As has been demonstrated for the erythropoietin receptor (EpoR) (Constantinescu et al. 2001, Remy et al. 1999), EGF-R (Martin-Fernandez et al. 2002, Moriki et al. 2001) and melatonin receptor 1 and 2 (Ayoub et al. 2002), these preformed complexes are activated through a ligand-induced conformational change.

Once dimerised, it is likely that GHR receptors stay associated during transport to the cell surface. The presence of GHR dimers at the plasma membrane offers an advantage for rapid signalling. First, low ligand concentrations might still be able to initiate receptor signalling, which is especially advantageous in cells with low GHR levels; second, no time is lost in recruiting a second receptor to the GH-GHR complex (Cunningham et al. 1991).

Except for homo-dimerisation, the ER does not seem to play a role in GHR surface expression. Pulse-chase experiments showed that most, if not all, of the GHR initially synthesised in the ER matures into complex-glycosylated 130-kDa polypeptides. Use of proteasom inhibitors to prevent degradation of mal-folded intermediates did not change the amounts of precursor and mature GHRs, nor did they effect the maturation rate of the GHR (van Kerkhof et al. 2000a). Thus the ER does not seem to play a regulatory role in the expression of GHR at the cell surface. This is in distinct contrast to the situation for another important cytokine receptor, the EpoR. The N-terminal 32–58 residues of the JH7 domain of Jak2, not of Jak1, bind to the EpoR in the ER and promote its cell surface expression. A continuous stretch of amino acids in the EpoR cytosolic tail is required for functional, ligand-independent binding to Jak2 and cell surface receptor expression, whereas four specific residues are essential in switching on pre-bound Jak2 after ligand binding. Thus, in addition to its kinase activity required for cytokine receptor signalling, Jak2 is also an essential subunit required for surface expression of EpoR (Huang, et al. 2001). No role for Jak2 in ER to Golgi transport of the GHR was obvious from experiments with $Jak2^{-/-}$ mouse embryonic fibroblasts in which we expressed the GHR (Alves dos Santos and Strous, unpublished observation).

2.3 GHR Traffic Control at the Plasma Membrane

At the cell surface the expression of GHR is controlled by two major factors: (a) endocytosis and (b) proteolytic cleavage of the extracellular domain. In the first process the clathrin-mediated endocytosis of the GHR depends on the activity of the ubiquitin system, and the second factor is theactivity of tumour necrosis factor-α-converting enzyme (TACE), also referred to as Adam17. Together, they determine for more than 90% the residence time of the GHR at the cell surface. Inhibition of both activities prolongs the half-life of the GHR from less than 1 h to several hours. Thus inhibition of both GHR endocytosis and degradation at the cell surface renders the cells several-fold more sensitive for GH (van Kerkhof et al. 2003).

In a Chinese hamster cell line (ts20) with a temperature-sensitive mutation in the ubiquitin-activating enzyme E1, expressing the wild-type GHR, it was shown that endocytosis and degradation of the GHR are dependent on a functional ubiquitin conjugation system. Poly-ubiquitination of the GHR is enhanced upon binding of GH at the permissive temperature and completely inhibited at the non-permissive temperature (Strous et al. 1996). Examination of truncated receptors, the endocytosis-deficient receptor mutant F327A, and conditions under which clathrin-mediated endocytosis is inhibited shows that GHR ubiquitination and internalisation are coupled events (Govers et al. 1997). Surprisingly, the internalisation of a truncated receptor, GHR (1–399; K271–362R), which is not ubiquitinated because all cytoplasmic lysine residues (i.e. the acceptor sites for ubiquitin) are replaced with arginine residues, still depends on a functional ubiquitin conjugation system (Govers et al. 1999).

A specific domain in the GHR cytoplasmic tail regulates receptor endocytosis via the ubiquitin conjugation system. This domain, the ubiquitin-dependent endocytosis motif (UbE motif), consists of the amino acid sequence DSWVEFIELD (Govers et al. 1999). In addition to the UbE motif, the cytoplasmic tail of the GHR contains a di-leucine motif at position 347. Upon truncation of the receptor at amino acid residue 349, this di-leucine motif is exposed and mediates ubiquitin-system-*independent* internalisation of the GHR. Similar to wtGHR, di-leucine-mediated GHR internalisation requires functional clathrin-coated pits and results in GHR transport to the lysosome. However, the full-length GHR internalises independent of the di-leucine motif (Govers et al. 1998).

The ubiquitin-proteasome degradation pathway provides the major pathway for non-lysosomal degradation (reviewed in Hershko and Ciechanover 1998). It is involved in the degradation of cytoplasmic proteins and proteins, which do not pass the quality control of the ER, and plays a role in regulating a variety of cellular functions (Plemper and Wolf 1999). Degradation is initiated by the ubiquitin conjugation system, through which poly-ubiquitin moieties are attached to cytoplasmic proteins, after which the modified proteins are recognised and degraded by a multi-subunit protease, the 26S proteasome (Thrower et al. 2000). Recently, it became evident that for a restricted number of plasma membrane proteins, ubiquitination triggers internalisation and vacuolar/lysosomal rather than proteasomal degradation (reviewed in Strous and Govers 1999). This pathway is best understood in yeast, where a number of plasma membrane proteins is endocytosed in an ubiquitin-dependent manner (Galan et al. 1996; Hicke 2001; Kölling and Hollenberg 1994; Terrell et al. 1998).

Endocytosis of the GHR occurs via the clathrin-mediated pathway but is regulated via the ubiquitin-proteasome system (Strous and van Kerkhof 2002) and requires an intact UbE motif (Govers et al. 1999). The formation of clathrin-coated pits at the plasma membrane and the recruitment of cargo proteins involve a network of proteins called adaptors, which mediate binding between cargo proteins and clathrin. Adaptors in clathrin-coated pit formation at the plasma membrane are (a) monomeric arrestins and (b) the heterotetrameric adaptor complex 2 (AP2). Non-visual arrestins are involved in the internalisation of a number of G protein-coupled receptors. For example, after ligand stimulation, the activated β2-adrenergic receptor is recruited by β-arrestin into clathrin-coated pits (Goodman et al. 1996, Santini et al. 2002). β-Arrestin can bind with its C-terminus to clathrin as well as to AP2 (Krupnick et al. 1997, Laporte et al. 1999). It was therefore suggested that the interaction with AP2 is required to cluster the β2-adrenergic receptor in clathrin-coated pits (Laporte et al. 2000).

The heterotetrameric adaptor complex AP2 belongs to a family of adaptor complexes consisting of AP1, AP2, AP3 and AP4 (for review see Kirchhausen 1999). All these complexes consist of two large subunits of about 100–130 kDa (α and β2 in AP2), a central subunit of 50 kDa (μ2 in AP2) and a small subunit around 20 kDa (σ2 in AP2). At the plasma membrane AP2 can function as a bridge between cargo membrane proteins and the clathrin coat. The interaction with clathrin is mediated by its β2 subunit and was narrowed down to a so-called "clathrin box", which is conserved amongst a number of clathrin-interacting proteins

(Gallusser and Kirchhausen 1993, Terhaar et al. 2000). Correct trafficking of membrane proteins depends on signal sequences or sorting signals. Interaction of cargo with AP2 generally depends on the presence of short peptide motifs of four to six amino acid residues present in the cytoplasmic tail (reviewed in Bonifacino and Traub, 2003).

The first sorting signal required for endocytosis was identified in the LDL receptor and consists of a peptide NPXY (X representing any amino acid). This motif is necessary and sufficient to mediate endocytosis (Chen et al. 1990) and interacts with the μ2 subunit of AP2 (Boll et al. 2002). More widely used is the YXXØ motif (Ø stands for a bulky hydrophobic side chain), which was first identified in the transferrin receptor (TfR) (Collawn et al. 1990). Support for the idea that YXXØ motifs interact with AP2 came from studies in which ligand-stimulated EGF-R coimmunoprecipitated AP2; this interaction depended on the presence of the YXXØ motif (Sorkin and Carpenter 1993; Sorkin et al. 1996). In yeast-two hybrid screens, it was shown that YXXØ motifs interact with the μ2 subunit of AP2 (Ohno et al. 1995). For the interaction of the μ2 subunit with EGF-R, the amino acid residues D176 and W421 of μ2 are crucial. Also, for TfR internalisation interaction with AP2 via the μ2 subunit is a pre-requisite. For the chicken GHR an interaction between α-adaptin and the GHR has been shown upon hormone stimulation (Vleurick et al. 1999). Considering that GH triggers neither mouse nor rabbit GHR endocytosis, this observation is in line with our observations that GHR endocytosis is K^+ dependent and that only endocytosis-competent GHR is present in clathrin-coated pits by immunogold electron microscopy (Govers et al. 1997, Sachse et al. 2001). However, attempts to find an association between AP2 and GHR failed, probably because of competition between factors of the ubiquitin system and AP2 (Schantl and Strous, unpublished data). In other membrane cargo proteins, for example, the CI-MPR and CD4, acidic di-leucine motifs mediate endocytosis (Glickman et al. 1989, Shin et al. 1991). This motif binds to the β2 subunit of AP2 (Rapoport et al. 1998). Proteins containing this motif do not compete in vivo for endocytic uptake with those containing a YXXØ motif, suggesting that they use distinct pathways, both depending on AP2 (Marks et al. 1997).

In the past few years, it has been demonstrated for an increasing number of yeast plasma membrane proteins that ubiquitination is required for their internalisation. Several studies showed that mono-ubiquitination of proteins is sufficient to stimulate their endocytosis (Galan and Haguenauer-Tsapis 1997; Roth and Davis 2000; Terrell et al. 1998). In mammalian cells, the GHR, the β-adrenergic receptor and the epithe-

lial sodium channel, ENaC, are the only cell surface proteins known which endocytose ubiquitin system-dependently (Shenoy et al. 2001; Staub et al. 1997; Strous and Gent 2002). Ubiquitin itself contains none of the above-mentioned sorting signals. Moreover, no ubiquitin moiety needs to be attached to the GHR at the time of endocytosis (Govers et al. 1999), whereas GHR endocytosis is completely blocked if the ubiquitin-activating enzyme E1 is inactivated (Strous et al. 1996). In yeast, endocytosis can occur independent of the AP2 orthologue (Huang et al. 1999). Therefore, it was suggested that two surface patches on the folded ubiquitin contain the information for internalisation of the ubiquitinated protein and may mediate the binding to the adaptor protein Epsin to initiate endocytosis (Shih et al. 2002; Shih et al. 2000). In mammalian cells, ubiquitination of β-arrestin by the E3 Mdm2 is required for endocytosis of the β2-adrenergic receptor, suggesting that the ubiquitination of an adaptor protein triggers endocytosis (Shenoy et al. 2001). Recently, accessory proteins involved in the primary steps of endocytosis, Eps15, Eps15R and Epsin, all three of which can bind clathrin as well as AP2, were shown to be mono-ubiquitinated (Klapisz et al. 2002; Polo et al. 2002; van Delft et al. 1997). The ubiquitination of these proteins suggests a regulatory function of ubiquitin in the endocytic machinery. Recently, the small glutamine-rich tetratricopeptide repeat-containing protein (SGT) was identified as a possible co-factor in GHR trafficking (Schantl et al. 2003). In Fig. 3 an overview of membrane protein cargo trafficking is depicted.

Taken together, four features render the GHR unique compared with other receptors that signal from the cell surface:

1. *Recruitment of the GHR into the coated pits depends on an active ubiquitin system*. This feature determines the average residence time at the cell surface. There is ample evidence from many clinical studies that in stress conditions the number of GHRs is decreased, because of rapid endocytosis (Frank 2001). A key role for the ubiquitin system in GHR internalisation emerges from both genetic and molecular experiments showing that GHR molecules accumulate at the plasma membrane if the ubiquitin system is inhibited (Strous and Govers 1999). Moreover, GHR ubiquitination coincides with its recruitment into clathrin-coated pits (van Kerkhof et al. 2000b). Strikingly, ubiquitination of the GHR itself is not required because replacement of all lysine residues by arginines in the GHR cytosolic tail does not inhibit internalisation (Govers et al. 1999). We have identified the target of the ubiquitin system in the GHR cytosolic tail as a 10-amino acid-long sequence, DSWVEFIELD,

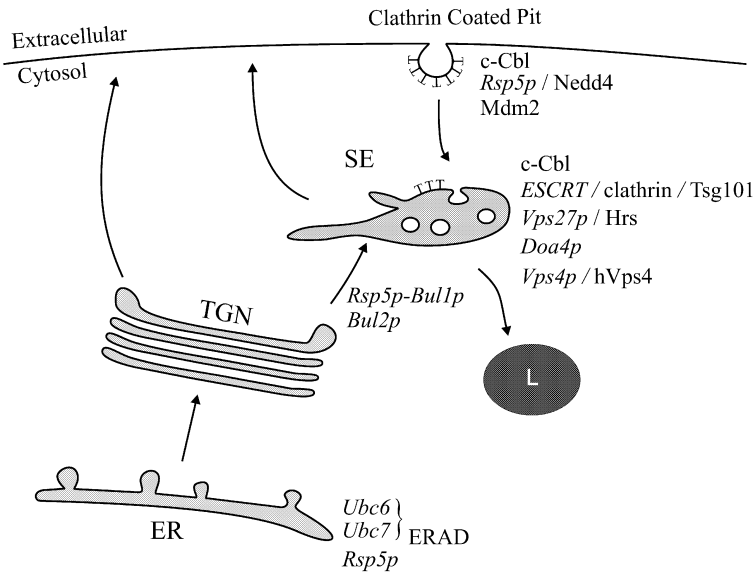

Fig. 3. Modulation of membrane proteins by the ubiquitin system at different intracellular locations. In the process of ER associated degradation (*ERAD*), malfolded proteins are recognized in the ER, translocated back into the cytosol, ubiquitinated (involving the E2 enzymes Ubc6 and Ubc7) and degraded by the 26S proteasome. At the *trans*-Golgi network the yeast HECT E3 Rsp5p acts together with Bul1p and Bul2p to direct the general amino acid permease (Gap1p) to a prevacuolar compartment [the yeast equivalent of the sorting endosome (*SE*)] instead of the plasma membrane (Soetens et al. 2001; Helliwell et al. 2001). Membrane proteins at the cell surface are often modified by covalent ubiquitin attachment. E3s involved in this process are indicated. Upon arrival in the SE, membrane proteins that have to be degraded in the vacuole/lysosome (*L*) are sorted away from proteins that recycle back to the plasma membrane. Sorting of cargo membrane proteins into the internal vesicles of the SE is mediated via the ubiquitin system and depends on the recognition of ubiquitinated proteins by proteins like Hrs, Tsg101 or the yeast ESCRT complex. Before inward budding is completed, ubiquitin moieties are removed from the ubiquitinated proteins by deubiquitinating enzymes such as yeast Doa4p, and the coat is disassembled by the AAA-ATPase Vps4p (Sachse et al. 2003). Finally, the membrane proteins are degraded by vacuolar or lysosomal protease. Yeast proteins are in *italics*

designated as the UbE motif for ubiquitin-dependent endocytosis (Govers et al. 1999). Besides the ubiquitin conjugating system, the 26S proteasome is also involved in GHR downregulation. Proteasome inhibitors prevent both internalisation of the GHR and endosome-to-lysosome transport (van Kerkhof et al. 2001; van Kerkhof et al. 2000a). The

inhibitory effect is lost when the GHR is truncated beyond amino acid 369, suggesting that an associated protein must be degraded by the proteasome before internalisation can occur (Sachse et al. 2002; van Kerkhof et al. 2000b).

2. *Endocytosis of the GHR is constitutive and ligand independent*. Thus cell surface expression of the GHR is not regulated by its ligand; upon arrival at the cell surface from the ER/Golgi complex, the GHR is recruited into coated vesicles (like cargo receptors such as the LDL receptor) and transported to lysosomes for degradation (unlike cargo receptors, which release their cargo in endosomes and immediately return to the plasma membrane for the next round of endocytosis) (van Kerkhof et al. 2002). One exception to this rule constitutes the GHR in the human B-lymphoblast cell line IM-9. These cells show a GH accelerated receptor downregulation (Ilondo et al. 1986; Saito et al. 1994). Whether this is a feature induced by Epstein-Barr virus transformation, or by a mutation in the GHR gene, is unknown.
3. *Only dimerised GHR is recruited into the coated pits by the ubiquitin system.* Curiously, the UbE motif is only an effective ubiquitin system target if the GHR is in a dimeric state: Replacement of the GHR extracellular domain by a part of the extracellular domain of LRP prevents GHR dimerisation and disqualifies the ubiquitin system as a regulator of GHR endocytosis (Gent et al. 2002).
4. *The ubiquitin system regulates GH sensitivity of cells.* Until now only the interaction between the tumour suppressor p53 and the E3 Mdm2 seems to be a direct protein-protein interaction independent of phosphorylation, implying that the concentration of these proteins in cytosol and/or nucleus is a major factor in controlling cellular life and death. Whether availability of GHRs at the cell surface is subjected to the same kind of mechanism remains to be elucidated. There is a remarkable difference between the involvement of the ubiquitin system in GHR trafficking and in other membrane proteins: In most, if not all, systems reported, the activity of the ubiquitin system is preceded by protein phosphorylation. In yeast, uptake of Ste2p and most permeases requires phosphorylation of serine or threonine before action of Rsp5p initiates uptake (De Craene et al. 2001; Hicke et al. 1998). In mammalian cells, uptake of ENaC requires serine phosphorylation before the HECT domain of Nedd4 can bind to the tetrameric sodium channel. Degradation via the RING motif E3 βTrCP requires previous phosphorylation as documented for NFκB, β-catenin, HIV-1 protein vpu, cyclins and myo-D, whereas c-Cbl stimulates degradation of RTKs only if their kinase domain is active. As the signal transduction pathway of the GHR via Jak2 is not involved in ubiquitin system-dependent endocytosis

(Alves dos Santos et al. 2001), and no other phosphorylation event has ever been observed, it may be that GHR internalisation relies on the ubiquitination of a GHR-associated protein which either is constitutively phosphorylated or directs the GHR into the endocytic pathway in a phosphorylation-independent way.

2.4 GHR Traffic Control in Endosomes

Once recruited into a coated pit via factors of the ubiquitin system and endocytic machinery (e.g. AP2, Eps15, Epsin, clathrin, dynamin) the GHR becomes a cargo molecule in the early endosome (Fig. 3). At this point the system has two major options for cargo membrane proteins: (a) the default recycling route back to the cell surface and (b) a second sorting step for degradation in the lysosomes. The recycling pathway is common for receptors involved in uptake and scavenging of proteins from the circulation like LDL, asialoglycoproteins, α-macroglobulin and apo-E. These receptors (such as LDL receptor, asialoglycoprotein receptor, LRP) release their soluble cargo molecules in the acidic environment of the endosome (Yamashiro et al. 1984). The low pH is generated by the vacuolar ATPase and is crucial for sorting of ligands that show a pH-dependent affinity towards their receptors (Johnson et al. 1993, Schmid et al. 1989). The receptors return to the cell surface, while their cargo (ligands) remains in the lumen of the vacuole and is transported to lysosomes for degradation (Geuze et al. 1983). In the case of the TfR, the acidic pH causes iron to dissociate from the receptor ligand transferrin, after which the apoTf-TfR complex recycles to the plasma membrane (Dautry-Varsat et al. 1983; Klausner et al. 1983). For lysosomal degradation (option b) membrane proteins must be identified by the ESCRT system which is located in specialised coated membrane domains of the sorting endosomes (SEs) (Sachse et al. 2002). Among these cargo membrane proteins are many growth factor receptors whose ligand binding is stable at the lower pH values of the SE. A common localisation throughout the endocytic pathway was shown for EGF and EGF-R and, similarly, a pool of intact PDGF-PDGF-R and NGF-TrkA (nerve growth factor) receptor complexes could be detected in endosomes (Sorkin and Waters 1993). As sorting into the internal vesicles of SEs depends on several actors of the ubiquitin/proteasome system (discussed below), this step is easily inhibited by proteosome inhibitors. We have shown that the degradation of NGF, internalised via its receptor TrkA, is inhibited

in the presence of proteasome inhibitors. Proteasome inhibitors were also shown to inhibit the degradation of the PDGF-R (Mori et al. 1995), the EGF-R (Levkowitz et al. 1998) and the Met tyrosine kinase receptor (Jeffers et al. 1997). RTKs are probably all sorted into the SE vesicles invaginating from the limiting membrane together with their ligands (Sachse et al. 2002). Also, for cytokine receptors it was indicated that proteasome inhibitors inhibit the downregulation at the SE (Martinez-Moczygemba and Huston 2001, Rocca et al. 2001; Verdier et al. 2000; Yen et al. 2000; Yu and Malek 2001). Notably, whereas membrane cargo proteins depend on ESCRT and the ubiquitin-proteasome system, soluble proteineous cargo and solutes travel via the same route by default (van Kerkhof et al. 2001).

Depending on the cell type, the vacuolar part of SEs is reached by endocytic tracers within 1–5 min (Kleijmeer et al. 1997; Schmid et al. 1988). By electron microscopy the irregularly shaped vacuole (250–500 nm) is electron-lucent with only a few internal vesicles. Internal endosomal vesicles are formed by inward budding of the limiting membrane, a process called micro-autophagy (Geuze 1998; Hopkins et al. 1990) (Fig. 3). In addition, SEs can bear a prominent clathrin coat (Holtzman and Dominitz 1968; Raposo et al. 2001; Sachse et al. 2002). Besides clathrin, coatomer complex I coats have been found on SEs, with a possible role in sorting of proteins towards lysosomal degradation (Gu et al. 1997; Piguet et al. 1999; Whitney et al. 1995).

The molecular mechanism for sorting the GHR into the SE internal vesicles remains largely unsolved. Unlike the RTKs, tyrosine kinase activity of recruited Jak2 is not required (Alves dos Santos et al. 2001). In RTKs both the ubiquitin ligase c-Cbl and the hepatocyte growth factor-regulated tyrosine kinase substrate Hrs act as intermediates in cargo selection by the ESCRT complex. For these events, the RTKs must be activated and the cytosolic tails must bear both phosphate residues and (mono)ubiquitin to accommodate the two factors. Hrs contains a clathrin-binding domain, localises to the flat clathrin lattices of SEs and binds directly to ubiquitin via its ubiquitin-interacting (UIM) motif (Raiborg et al. 2002; Sachse et al. 2002; Urbé et al. 2000; Urbé et al. 2003). GHR selection into the SE internal vesicles does not involve c-Cbl; it requires the general co-factors of ESCRT, Hrs and the AAA-type ATPase hVPS4 but does not require ubiquitination of the receptor (Govers et al. 1999; Sachse et al. 2003; van Kerkhof et al. 2001; van Kerkhof et al. 2000b).

The question then is, what is required for the GHR to pass the sorting step in the SE? As GHR endocytosis depends on a functional ubiquitin

conjugation system, an intact UbE motif and active proteasomes, the answer is not easily obtained. Most of the experimental approaches use one of the three variables, conditions that inhibit GHR entry into the cells. To address this question, a GHR was truncated at position 349 of the cytosolic tail. In this truncated GHR a di-leucine motif is activated that mediates internalisation in an ubiquitin system- and proteasome-*independent* fashion (Govers et al. 1998). Mutation of phenylalanine residue 327 to alanine in the UbE motif abolished ubiquitination of both full length- and truncated GHR (Govers et al. 1999) but did not influence the internalisation of the truncated GHR(1–349). Both pulse chase and ^{125}I-GH uptake experiments showed that an intact UbE in the truncated GHR(1–349) is required for efficient degradation of both ligand and receptor (van Kerkhof et al. 2001). As the degradation of the ligand occurs in lysosomes (Murphy and Lazarus 1984; Yamada et al. 1987), the conclusion is that the UbE motif is required for endosome to lysosome sorting of both receptor and ligand and that the C-terminal di-leucine motif intermediary in endocytosis is not recognised by the cargo selectors at the SE. Thus the UbE motif is required both at the cell surface and at the SE for correct sorting and presumably the same factors of the ubiquitin conjugation system are involved. Analogous to the situation for EGF, PDGF and CSF-1 receptors, in which c-Cbl seems to initiate cargo selection at the SE by adding ubiquitin to the (phosphorylated) receptors (Joazeiro et al. 1999; Lee et al. 1999; Levkowitz et al. 1999; Miyake et al. 1998), all indications point to a ubiquitin ligase-specific for *dimerised* GHRs as initiator for cargo selection. The E3 for GHR sorting is unknown yet, but it is probably not c-Cbl as the GHR does not require GH-activation and Jak2 activity for this step. Very likely it will be the same E3 as required for GHR endocytosis. The E3-GHR complex might then be recognised by Hrs and incorporated into the ESCRT system. Whether ancillary factors are involved or other modifications are needed remains to be investigated. An interesting observation in this respect is that truncation of the cytosolic tail precedes degradation of the luminal domain (van Kerkhof and Strous 2001).

The idea that the same ubiquitination factors are involved at the cell surface and at the SE is strengthened by the observation that at both sites ubiquitination of the GHR per se is not needed, only competent cellular ubiquitination machinery and the UbE motif within the GHR cytosolic domain (Govers et al. 1999; van Kerkhof et al. 2001). This suggests that, in this system, ubiquitination of another component serves as cargo selection signal for the receptor, that ubiquitination and subsequent degradation of an inhibitory molecule promote exposure of a ubiquitin-

independent sorting signal or that the docked ubiquitination machinery itself targets the complex for uptake. An example of the last possibility is the recent discovery that CIN85 bridges a receptor-bound E3 ligase and endophilin, a known endocytic protein, to promote internalisation (Petrelli et al. 2002; Soubeyran et al. 2002).

The involvement of proteasomes in the molecular mechanism of cargo packaging either at the cell surface or at the level of SEs is presently unclear. The half-life of many membrane proteins and growth factor receptors is increased twofold or more in the presence of proteasome inhibitors. We investigated the role of the ubiquitin-proteasome pathway in the degradation of GH and GHR(1–349) by using the peptide aldehyde MG-132 as well as the highly specific clasto-lactacystin β-lactone (Craiu et al. 1997). Under these conditions, GHR(1–349) can enter the cells freely via the di-leucine motif. Degradation of both the GH and the GHR(1–349) was almost completely inhibited, indicating that proteasome activity is required for sorting from the SE to the lysosome (van Kerkhof et al. 2001). Also, lysosomal sorting of NGF endocytosed via TrkA was completely inhibited by proteasome inhibitors, whereas endocytosis of TrkA does not depend on the ubiquitin-proteasome system. Again, this indicates that SE-to-lysosome transport requires the activity of the ubiquitin conjugation system, and perhaps also of the proteasome. It is possible that there is no direct role for the proteasome, because use of proteasome inhibitors might exhaust the cells of free ubiquitin which might lead to reduced ubiquitination of the target protein and reduced endosomal sorting (Swaminathan et al. 1999; van Kerkhof et al. 2001).

The question remains: What is the target for the proteasome? It is clear that shortly after endocytosis the degradation of the GHR starts at the cytosolic tail before it reaches the lysosomes (van Kerkhof and Strous 2001b). Whether this cytosolic degradation is due to proteasome activity remains unclear. A crucial question is whether cytosolic degradation is a requisite for cargo selection by the ubiquitin and the ESCRT system. The di-leucine motif probably does not play a role in this sorting event because its mutation does not affect the effective transport to lysosomes and degradation of GH (Govers et al. 1998). The conserved DSGRTS sequence between amino acid residues 365 and 370 is interesting, as similar sequences (consensus DSGxxS) in β-catenin and IκBα are targeted by specific kinases, after which the E3 βTrCP poly-ubiquitinates the two proteins (Aberle et al. 1997; Chen et al. 1995). Whether partial degradation of GHR plays a role in endosome to lysosome sorting via the DSGxxS motif remains to be investigated. A complicating factor is

the observation that ubiquitination of the GHR is not required for proper sorting.

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

3
GHR Signalling Control

3.1
GH Binding to GHR

GH binding to the GHR is the first step in the signalling cascade. With crystallography and gel filtration, a single GH molecule was shown to form a ternary complex with two GHR extracellular domains (Cunningham et al. 1991; de Vos et al. 1992). Two distinct binding sites were found in GH: a high-affinity site 1 covering a surface area of about 1,230 $Å^2$ and a slightly lower-affinity site 2 of about 900 $Å^2$. Because of the different affinities, complex formation has been proposed to occur sequentially (Cunningham et al. 1991). Strikingly, the two distinct binding sites of GH interact with the same amino acids located in the N-terminal fibronectin type III domain (sub-domain 1) of the GHR molecules, suggesting that no more than one GH molecule can bind to a single GHR. Recruitment of the second GHR to the complex is a prerequisite for signal transduction as was demonstrated with a GH site 2 mutant. Mutation of the glycine at position 120 of human GH to arginine (G120R) or lysine (G120K) disrupts the binding via site 2 (Fuh et al. 1992). Because binding site 1 is unaffected, competition with endogenous GH for GHR molecules occurs, hence explaining the dwarf phenotype of transgenic mice overexpressing this GH site 2 mutant (Chen et al. 1990). In cell systems, the GH site 2 mutant antagonises the effects of GH when applied in a three to fivefold excess (Zhang et al. 1999). Combination of the G120K mutation with mutations which enhance the affinity of site 1 resulted in the potent GH antagonist B2036 (Maamra et al. 1999). When conjugated to polyethylene glycol to decrease glomerular

filtration in the kidney, this antagonist is effective in treatment of acromegalic patients (Trainer et al. 2000).

3.2
Signalling Events at the Cell Surface

GH binding to GHR induces the transcription of genes encoding for a variety of proteins including GHR, IGF-1, insulin, LDL receptor, serine protease inhibitor (spi) 2.1, cytochrome *P*450 and the transcription factors c-Fos, c-Jun and c-Myc (Carter-Su et al. 1996). The four known signalling pathways initiated by the GHR are depicted in Fig. 4. To initiate signalling, GHR recruits and transiently activates the cytosolic tyrosine kinase Jak2 (Argetsinger et al. 1993). This event takes place at the cell

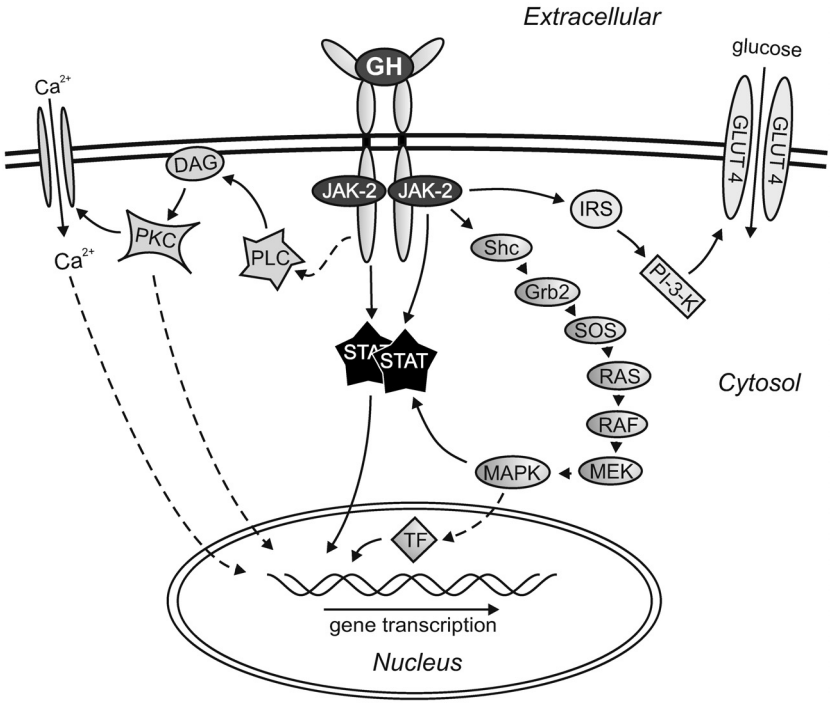

Fig. 4. The signalling pathways activated by GH. GH binding to GHR results in activation of the STAT, MAPK, IRS and/or PKC pathways which ultimately results in activation of gene transcription. See text for details. (Modified from Argetsinger and Carter Su 1996)

surface because Jak2 is also activated by GH under conditions that impair GHR internalisation (potassium depletion) (Govers et al. 1997; Alves dos Santos et al. 2001). Mutation or deletion of the proline-rich box 1 region impairs Jak2 binding (Sotiropoulos et al. 1994; VanderKuur et al. 1994). However, for maximal Jak2 activation the membrane-proximal one-third of the GHR cytoplasmic domain is required. According to the current model, GH induces a conformational change in the dimerised GHR that brings two Jak2 molecules in close proximity, thereby facilitating trans-phosphorylation of tyrosine residues in the kinase domain of the paired Jak2 (Gent et al. 2002). Subsequently, these activated Jak2 molecules phosphorylate tyrosine residues of the GHR (Wang et al. 1996) and signalling molecules (Carter-Su et al. 1996). In general, phosphorylated tyrosine residues serve as docking sites for proteins with Src homology 2 (SH2) or phosphotyrosine binding domains.

A direct pathway from activated GHR to gene transcription involves members of the signal transducers and activators of transcription (STATs) family, the *STAT pathway* (Darnell 1997). STAT proteins are recruited via their SH2 domain to the phosphorylated GHR (e.g. STAT5A and -5B) or to Jak2 (e.g. STAT1 and -3), are then phosphorylated on a conserved C-terminal tyrosine residue, homo- or heterodimerise with other STAT molecules, translocate to the nucleus, bind to specific DNA promoter sequences and activate transcription of several target genes. For example, STAT1 and -3 bind to sis-inducible elements (SIE) in the c-Fos promoter (Campbell et al. 1995), whereas STAT5 binds the interferon-γ-activated sequence (GAS)-like response element (GLE) in the spi2.1 gene (Wood et al. 1997). Recently, the mitogen-activated protein (MAP) kinases ERK1 and ERK2 have been implicated in the serine phosphorylation of STAT proteins (Pircher et al. 1999). Both kinases are also activated in response to GH via a complex cascade known as the *RAS-MAP kinase pathway*. MAP kinases are serine/threonine kinases, which are activated by dual phosphorylation on tyrosine and threonine residues and are important regulators of cellular growth and differentiation (Cobb and Goldsmith 1995). The signalling pathway starts with the binding of the Src homology-containing (Shc) protein to activated Jak2, followed by Shc phosphorylation (VanderKuur et al. 1995). The cascade continues via growth factor receptor-bound 2 (Grb2), son of sevenless (SOS), RAS, RAF and mitogen-activated/extracellular signal-regulated kinase (MEK) and ultimately results in the activation of MAP kinases ERK1 and ERK2 (VanderKuur et al. 1997). In addition to the STAT pathway, MAP kinases activate a variety of proteins including phospholipase A2, the ribosomal S6 kinase p90RSK, cytoskeletal proteins and tran-

scription factors like c-Jun, c-Myc and the ternary complex factor $p62^{TCF}$/ELK1 (Davis 1993).

GH binding also results in the tyrosine phosphorylation of insulin receptor substrate (IRS)-1, IRS-2 and IRS-3 (Argetsinger et al. 1995; Souza et al. 1994). These molecules were identified as signalling molecules for insulin and the closely related IGFs and are therefore proposed to mediate the insulin-like effects (e.g. glucose uptake and lipogenesis) of GH. Phosphorylated IRS proteins associate with the 85-kDa regulatory subunit of phosphatidylinositol (PI)-3-kinase (Ridderstrale and Tornqvist 1994). PI-3-kinase activation leads to translocation of the insulin-dependent glucose transporter GLUT-4 to the plasma membrane, thereby stimulating the uptake of glucose (Cheatham et al. 1994). Consistently, inhibition of PI-3-kinase by wortmannin blocks the insulin-like effects of GH in rat adipocytes (Ridderstrale and Tornqvist 1994).

A fourth signalling pathway which is activated upon GH stimulation involves the activation of protein kinase C (PKC) through phospholipase C (PLC) (Moutoussamy et al. 1998). Although the exact mechanism of PLC activation is unknown, the involvement of a G protein has been suggested. Upon activation PLC hydrolyses inositol phospholipids to generate inositol phosphates and 1,2-diacylglycerol (DAG), which acts as a potent activator of PKC. The IRS/PI-3-kinase pathway has also been implicated in PKC activation, but this may be GH independent and restricted to certain PKC isoforms (Argetsinger and Carter Su 1996). Activated PKC stimulates lipogenesis and c-Fos expression and increases intracellular Ca^{2+} levels by activating L-type calcium channels (Gaur et al. 1996a, Gaur et al. 1996b). Intracellular calcium is essential for GH-induced transcription of the spi2.1 gene (Billestrup et al. 1995). Currently, regulation of Ca^{2+} influx via L-type calcium channels is the only event known which is Jak2 independent.

3.3 Modulation of Signal Transduction

In addition to the molecules initiating gene transcription, several factors have been proposed to modulate the GHR signalling activity. Phosphorylated Jak2 recruits the cytosolic SH2 domain-containing protein SH2-B, which stimulates the kinase activity of Jak2 and enhances signal transduction (Rui and Carter-Su 1999). Tyrosine phosphatases have been implicated in downregulation of GHR-mediated signalling. In response to GH, the SH2-domain containing tyrosine phosphatase SHP-1 binds and dephosphorylates Jak2 (Gebert et al. 1999; Hackett et al. 1997). Also

SHP-2, an ubiquitously expressed tyrosine phosphatase homologous to SHP-1, has been demonstrated to bind directly to a phosphorylated tyrosine residue in the C-terminal region of the GHR (Stofega et al. 2000). Mutation of this tyrosine residue or deletion of the C-terminal region results in the prolonged activation of the GHR, Jak2 and STAT5 (Alves dos Santos et al. 2001; Stofega et al. 2000). SHP-2 can also act via an ancillary protein: GH stimulation induces tyrosyl phosphorylation of the transmembrane glycoprotein signal-regulatory protein SIRPα1, which then recruits one or more SHP-2 molecules (Stofega et al. 1998). Interaction between SIRPα1 and SHP-2 enhances the phosphatase activity of SHP-2 and results in the dephosphorylation of SIRPα1, Jak2 and possibly the GHR (Stofega et al. 2000).

Recently, a new family of suppressor of cytokine signalling (SOCS) proteins has been implicated in a negative-feedback mechanism on cytokine signalling (Starr et al. 1997). In addition to a 40-amino acid C-terminal region called SOCS-box, all SOCS proteins contain a central SH2 domain (Krebs and Hilton 2000). Of the eight known members, GH induces rapid expression of SOCS-1, -2 and -3 and cytokine-inducible SH2-domain containing protein (CIS) to different extents (Adams et al. 1998; Tollet-Egnell et al. 1999), probably mediated via STAT proteins (Matsumoto et al. 1997). The importance of regulating signal transduction via SOCS proteins is demonstrated by the giant phenotype of mice lacking SOCS-2 (Metcalf et al. 2000) and the dwarf size when CIS is overexpressed (Matsumoto et al. 1999). The SOCS proteins use variable mechanisms to inhibit signalling. SOCS-1 inhibits Jak2 kinase activity by directly binding to the kinase activating domain (Yasukawa et al. 1999). SOCS-3 binds to tyrosine residues in close proximity to box 1 and might therefore prevent Jak2 association with the GHR (Ram and Waxman 1999). CIS and SOCS-2 bind to phosphotyrosine residues in the C-terminal region of GHR and compete with STATs and/or other signalling molecules (Ram and Waxman 1999). Recently, an alternative model has been proposed, based on the observation that the conserved SOCS-box binds to the elongin B/C complex (Zhang et al. 1999). These elongins also interact with cullin-2, a putative ubiquitin-ligase, and thereby mediate the ubiquitination and subsequent degradation of the SOCS protein and its interacting molecules like Jak2 (Ungureanu et al. 2002). Consistent with this model, proteasome inhibitors prolong phosphorylation of Jak2 and the GHR (Alves dos Santos et al. 2001).

3.4
Jak2 Activity and GHR Trafficking Are Independent

The role of Jak2-mediated signal transduction in GHR membrane trafficking seems to be minor. For the EpoR, another cytokine receptor, the regulation of cell surface expression depends on Jak2. Jak2, not Jak1, binds to the EpoR in the ER and promotes its cell surface expression. Thus, for the EpoR, Jak2 is not only essential for its signal transduction; it also acts as a chaperone along the biosynthetic pathway to regulate its surface expression (Huang et al. 2001). Replacement of all four prolines in the box-1 of the GHR by alanine residues resulted in the complete absence of both receptor and Jak2 phosphorylation. This modification, however, did not alter the rate and extent of receptor-bound growth hormone internalisation compared with a functional GHR, nor did it change its turnover and transport to the plasma membrane (Alves dos Santos et al. 2001). In addition, the receptor was still normally ubiquitinated and remained dependent on both an intact ubiquitin system and proteasome action for its internalisation. These experiments warrant the conclusion that, although endocytosis and degradation require the action of the ubiquitin system, they are fully independent of Jak2-dependent GHR signal transduction (Alves dos Santos et al. 2001). In addition, Jak2-*independent* GH signalling is probably not involved in trafficking, because binding of GH to its receptor does not effect GHR endocytosis and degradation (van Kerkhof et al. 2002).

GHR endocytosis is inhibited in the presence of proteasome inhibitors (van Kerkhof et al. 2000a). In addition, the downregulation of signal transduction as measured by the extent of tyrosine phosphorylation is inhibited by proteasome inhibitors. The obvious explanation would be that downregulation of GHR signal transduction is via endocytosis and lysosomal degradation. This appears not to be the case: The proteasome inhibitor MG132 prolongs the GH-induced activity of both GHR and Jak2 through inhibition of GHR and Jak2 tyrosine de-phosphorylation (Alves dos Santos et al. 2001). This result was confirmed when proteasome inhibitor was combined with ligand in an endocytosis-deficient GHR mutant. Thus proteasome action on tyrosine de-phosphorylation is independent of endocytosis. In addition, the proteasome plays a role in downregulation of GHR signal transduction.

Which mechanisms underlie the signal transduction downregulation of the GHR and JAK2 proteins? Experiments with a truncated tail mutant, GHR(1–369) revealed prolonged Jak2 phosphorylation caused by the loss of a phosphatase-binding site. In erythropoietin-induced cellu-

lar proliferation, recruitment of SHP-1 accomplishes dephosphorylation of Jak2 and subsequent termination of signal transduction (Jiao et al. 1996, Klingmuller et al. 1995). A similar role for SHP-1 in mediating the downregulation of Jak2 after stimulation of cells with GH has been proposed (Hackett et al. 1997). Our results with 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. This pattern of prolonged phosphorylation is similar to that of Jak2 in full wild-type GHRs treated with proteasome inhibitor. One explanation might be that the phosphatase activity is modulated by proteasome function, perhaps by degrading an inhibitory complex in a similar manner as for the inhibitor of the transcription factor NF-κBα (Yaron et al. 1997). This scenario suggests a stabilised phosphatase inhibitor complex in the presence of MG132 which prevents the de-phosphorylation of the JAK2 by SHP-1, 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 (Piao et al. 1996), suggesting that the proteasome is involved in SHP-1 regulation.

3.5
Signalling Control at the Endosomes

Signalling of tyrosine kinase receptors, like TrkA, EGF-R and the insulin receptor, continues after endocytosis (Ceresa et al. 1998; Grimes et al. 1996; Vieira et al. 1996). What happens to the signal transduction of the GHR after its internalisation? This question is relevant because the average time span of GH-GHR complexes at the cell surface is roughly the same as the transport time between endocytosis and segregation into the SE internal vesicles (5 min). The nature and intensity of signal transduction might be different because of different sets of signal transduction factors (Fig. 4). 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 (Alves dos Santos et al. 2001). Preliminary estimation of the tyrosine-phosphorylated proteins indicates that the nature of the signalling inside does not differ from signalling from the cell surface. Co-immunoprecipitation with anti-GH and immunoblotting with antibodies against the GH-binding domain also showed that both full-length and truncated forms of the GHR are present in endosomes, indicating that the receptor is indeed degraded from the cytosolic tail shortly after en-

docytosis. 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 differ substantially. Notably, binding of ligand to its cognate receptor may activate signal transduction pathways, which continue after internalisation, probably from endocytic vesicles and endosomes (Alves dos Santos et al. 2001; Vieira et al. 1996; Wiley and Burke 2001). Incorporation of receptors into internal endosomal vesicles segregates them from signalling molecules in the cytoplasm and provides an efficient way to terminate signalling.

4 The Potential of the System

GH is an important regulator of cellular metabolism and acts via the GHR. The expression level and the residence time at the cell surface together determine the number of GHRs at the surface. Unlike most known growth factor receptors, the GHR is synthesised and degraded continuously with a half-life of less than 60 min. Our research data indicate that, once synthesised, the number of receptors at the cell surface is mainly regulated by ubiquitin system-dependent uptake and degradation. This process is specific and, among growth factor receptors, unique for the GHR. Exogenous conditions, such as starvation and cell stress, stimulate GHR degradation. In addition, the uptake (and degradation) of GHRs is regulated by the ubiquitin system only if they are dimerised. Another significant area of GHR research concerns cell differentiation and regulation of metabolism. Model systems are the fibroblast cell lines 3T3-F442A and Ob-1771, which can be induced to differentiate into cells that possess the biochemical and morphological characteristics of adipocytes. One of many ways to exert anabolic activities has recently been illustrated in 3T3-F442A cells: GH, by activating ERKs, can modulate EGF-induced EGFR trafficking and signalling and initiates cross-talk between the GH and EGF signalling systems (Huang et al. 2003). When 3T3 cells become fully differentiated they are no longer able to proliferate and in the end apoptosis is inevitable, offering a model system to study metabolism and apoptosis. A second cell line, which expresses relatively high levels of endogenous GHRs, is IM-9 lymphoblasts. These human cells express several Epstein-Barr viral membrane proteins. Strikingly, in IM-9 cells GHR endocytosis depends on GH binding. Thus IM-9 cells are not only a natural system to study im-

mune evasion mechanisms by Epstein-Barr virus, they will also facilitate study on the mechanism of GHR downregulation.

The merging the fields of cell biology and physiology will constitute a new interface for our insight in GHR function. Understanding the molecular principles of GHR trafficking as it is uniquely controlled by the ubiquitin-proteasome system will open up a new dimension in the understanding of how cells handle a multitude of signals to control cell differentiation, stress, apoptosis and anabolic and catabolic pathways.

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