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MODULATION OF GROWTH HORMONE RECEPTOR ABUNDANCE AND FUNCTION: ROLES FOR THE UBIQUITIN-PROTEASOME SYSTEM

Stuart J. Frank[1,2,3] and **Serge Y. Fuchs**[4]

[1] Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, 35294-0012

[2] Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, University of Alabama at Birmingham, Birmingham, AL, 35294-0012

[3] Endocrinology Section, Medical Service, Veterans Affairs Medical Center, Birmingham, AL 35233

[4] Department of Animal Biology, University of Pennsylvania, Philadelphia, PA 19104-6046

Summary

Growth hormone plays an important role in regulating numerous functions in vertebrates. Several pathways that negatively regulate the magnitude and duration of its signaling (including expression of tyrosine phosphatases, SOCS and PIAS proteins) are shared between signaling induced by growth hormone itself and by other cytokines. Here we overview downregulation of the growth hormone receptor as the most specific and potent mechanism of restricting cellular responses to growth hormone and analyze the role of several proteolytic systems and, specifically, ubiquitin-dependent pathways in this regulation.

Growth Hormone Actions and Regulation of Receptor Abundance

Growth hormone (GH) is a 22,000 Da peptide hormone derived mainly from the anterior pituitary gland that is critical in promotion of growth and regulation of metabolism and energy balance and may have a role in longevity in humans and other vertebrates [1,2]. Clinically, disruptions in GH action are most evident when either GH levels are either too high or too low. GH excess found in the setting of a GH-secreting pituitary adenoma yields the syndrome of acromegaly with its characteristic connective tissue and bony overgrowth, visceromegaly, and insulin resistance [3]; if present prior to closure of the epiphyseal growth plates, gigantism results. Conversely, GH deficiency in childhood yields shortness of stature, altered fat distribution, and a tendency to hypoglycemia [4]. In addition to these scenarios, an emerging experimental literature suggests potential roles for both pituitary-derived GH and autocrine/paracrine GH produced in extra-pituitary sites in potentiating or aberrantly promoting certain cancers [5–9].

Address correspondence to: Stuart J. Frank, University of Alabama at Birmingham, 1530 3rd Avenue South, BDB 861, Birmingham, AL 35294-0012, Tel. 205 934-9877, Fax 205 934-4389, E-mail – sjfrank@uab.edu.

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Observations in patients with GH resistance (Laron syndrome) [10] and analysis of mice with targeted deletion of the GH receptor (GHR) [11] indicate that the in vivo biological actions of GH are transduced by the GHR, a member of the cytokine receptor superfamily [12,13]. GHR is a widely distributed ~620 residue type 1 single membrane-spanning glycoprotein, expressed in many species, that likely exists as a dimer at the cell surface where it binds GH in its extracellular domain and signals by regulated interaction of its ~350 residue intracellular domain with signaling molecules [13–16]. The GHR couples physically and functionally the Janus kinase, JAK2, which is a cytoplasmic tyrosine kinase that is also utilized by other cytokine receptor family members [17,18]. JAK2 associates non-covalently with the dimerized GHR during its biogenesis and at the cell surface via the receptor's proline-rich perimembranous intracellular domain Box 1 element and the N-terminal FERM domain of JAK2 [19–25]. GH-triggered activation of JAK2 causes GHR and JAK2 tyrosine phosphorylation and induces signaling systems including STATs (most notably STAT5b), ERKs, and PI3-kinase [14–16] (Figure 1). GH-induced STAT5b activation requires receptor tyrosine phosphorylation and promotes gene transcription (eg., IGF-1, acid-labile subunit (ALS) of the IGF binding protein complex, SOCS proteins, hepatic P450 enzymes, and serine protease inhibitor 2.1 (Spi2.1) [26–36]). Unlike STAT5b, GH-induced ERK and PI3K activation do not require the entire GHR cytoplasmic domain, but only JAK2 coupling [21–23,37–39]. ERK activity is critical for GH-induced c-fos transcription [40], enhances GH-stimulated proliferation [41], and mediates crosstalk with EGF signaling [42–44]. GH-induced PI3K activity is implicated in anti-apoptosis and/or proliferation and likely contributes to GH-induced ERK, p70 S6 kinase, and phosphodiesterase activity [40,41,45–49].

GH sensitivity is substantially affected by the abundance of GHR available for ligand engagement at particular target cells and tissues. Surface GHR availability is regulated at several levels, including transcriptional, post-transcriptional, and post-translational. Transcriptional and post-transcriptional GHR regulation have been reviewed extensively [50–52] and will not be further dealt with in this review. Post-translational regulation of GHR abundance is exerted at the levels of receptor biosynthesis and trafficking to the cell surface, stability and constitutive (non-GH-dependent) downregulation of the surface receptor, metalloprotease-mediated GHR processing, and GH-induced GHR downregulation. Elements of each of these processes are impacted upon by the ubiquitin-proteasome system and each will be further discussed below in this context.

The ubiquitin-proteasome system is critical in maintenance of cellular physiology and the health of humans and other organisms. With regard to GH action and the GH axis, there are as yet no examples of specific disruptions of the ubiqutin-proteasome system that themselves account for disease states, but two interesting examples of mutational alteration of GH signaling elements that affect protein turnover in disease states have recently been defined that may relate to this important system.

One of these pertains to the syndrome of acromegaly. As alluded to above, the basis for this syndrome is in almost all instances a somatotroph adenoma that secretes excessive GH in a dysregulated fashion. Nearly twenty years ago, investigators uncovered a pituitary-specific activating mutation of the G-protein that couples to the GH releasing hormone receptor, thereby underlying the pathogenesis of many so-called "densely granulated" GH-secreting anterior pituitary adenomas [53–56]. A recent analysis of pituitary tumor specimens also demonstrated that nearly one-half of densely granulated tumors harbored such an activating mutation, but none were found in any of the fourteen "sparsely granulated" GH-secreting tumors examined [57]. Interestingly, six (43%) of these sparsely granulated tumors harbored a heterozygous mutation of the GHR that predicts a change of His to Leu in codon 49 of the extracellular domain; none of the densely granulated tumors exhibited any mutations of GHR. Functional analysis of a site-directed H49L mutant GHR in a reconstitution system (more below) revealed

that this mutant receptor exhibited altered posttranslational processing with accumulation of the precursor form of the receptor at the expense of the mature form. Notably, however, the mutant precursor was markedly longer-lived than the wild-type precursor and the mature form of the mutant that found its way to the cell surface displayed decreased GH binding capacity and diminished GH signaling [57]. This work is important in that it suggests an autofeedback loop of GH on the anterior pituitary somatotrophs via the somatotroph GHR, the disruption of which leads to hypersecretion of GH; this is a novel concept in pituitary-GH physiology. Furthermore, it will be important to determine if the buildup of the precursor (which, as seen below, is normally either rapidly processed to the mature form or undergoes endoplasmic reticulum-associated degradation) is due to or causative of disruption of the ubiquitin-proteasome system in these cells.

Another recent example of alteration of a GH signaling component relating to the function of the ubiquitin-proteasome system derives from investigation of a naturally-occurring mutation of STAT 5b [58,59]. Targeted deletion of Stat 5b in mice leads to disruption of key GH-dependent growth and metabolic functions [34]. Kofoed, et al [59] described a young woman with a homozygous mutation of the Stat 5b gene that predicts a change of alanine to proline at residue 630 in the phosphotyrosine-binding SH2 domain. This patient had shortness of stature and evidenced clinical and biochemical GH insensitivity. When the mutation was studied in the context of cellular reconstitution experiments [58], this A630P Stat 5b mutant was found to have a dramatically shortened half-life and failed to undergo activation in response to GH. Furthermore, the mutant displayed markedly enhanced aggregation and formed cytoplasmic inclusions. Perhaps most notably, expression of the mutant Stat 5b conferred defective proteasome function on the cells in which it was expressed, such that other proteins normally degraded by the proteasome were less susceptible to this activity. Thus, mutation of this critical GH signal transducing element not only caused GH resistance, but also disrupted proteasomal function because of its aberrant folding.

Trafficking of the Newly Synthesized GHR: Roles of JAK2 and the Proteasome

The GHR is synthesized as a non-glycosylated nascent precursor that is transported from the endoplasmic reticulum (ER) to the Golgi apparatus. It is now clear that GHR dimerizes in the ER early in the process of biogenesis, thus accounting for the receptor dimers detected at the cell surface even in the absence of GH engagement [19,60,61]. Recent studies undertook to determine the role, if any, that association of the GHR with JAK2 has on the receptor's trafficking to the cell surface [62,63]. These studies yielded not only an appreciation of JAK2's role as a potential chaperone early in the biosynthetic pathway, but also revealed an interesting role for the proteasome with regard to GHR trafficking.

As for many other surface glycoprotein receptors, the progress of GHR biogenesis and trafficking can be tracked by monitoring biochemically the degree and type of glycosylation it undergoes. In the process of transport through the Golgi, the GHR acquires carbohydrate in a characteristic fashion, in which high-mannose sugars added in the ER are ultimately removed during the transition from the early to late Golgi to yield the mature glycosylated GHR that populates the cell surface. This process can be assessed by determining the receptor's sensitivity to in vitro deglycosylation by endoglycosidase H (endoH). Mature GHR is endoglycosidase H (endoH)-resistant while the high-mannose GHR precursor is sensitive to deglycosylation by endoH [25,30,62,64,65]. Using this feature, as well as a robust cellular stable reconstitution system in which GHR was expressed either in the presence or absence of JAK2 and immunoblotting and pulse-chase metabolic labeling techniques, it was first observed that although JAK2 is not required for detectable surface GHR expression, cells expressing JAK2 exhibited enhanced surface GHR abundance and an increased mature:precursor GHR

ratio compared with JAK2-deficient cells, suggesting that JAK2 fosters GHR maturation [25,62].

Furthermore, in cells that lack JAK2, a GHR fragment was detected with antibodies to the receptor intracellular domain, but not the extracellular domain [63,66]. The abundance of this fragment and the steady state level of GHR precursor relative to the mature form were dramatically lessened by expression of wild-type JAK2 or JAK2 mutants that were capable of interacting with GHR, independent of whether such mutants possessed tyrosine kinase activity. Similarly, cells that harbored a GHR mutant that lacks the ability to interact with JAK2 (by virtue of mutation of the receptor Box 1 region) also manifested the same intracellular domain-containing GHR fragment and a decreased mature:precursor GHR ratio, independent of whether JAK2 was expressed. These data point to the ability of GHR to associate with JAK2 as enabling efficient receptor maturation and indicate that the presence of the intracellular domain-containing fragment of the receptor reflects inefficiency in this process.

Further studies focused on factors that affected the level of the intracellular domain-containing fragment in JAK2-deficient cells [63]. Notably, treatment of these cells with a proteasome inhibitor (lactacystin), but not two separate lysosome inhibitors, dramatically decreased the level of this receptor fragment and this was accompanied by decreased precursor GHR and increased mature GHR abundance. When brefeldin A (BFA) was used to disrupt ER-to-Golgi transport, the abundance of the intracellular domain fragment was also reduced; washout of BFA allowed regeneration of the fragment along with the GHR precursor. Interestingly, washout of BFA in the presence of cycloheximide (to prevent new protein synthesis) blocked reappearance of both the intracellular domain fragment and the precursor GHR, but washout of BFA in the presence of lactacystin prevented reappearance only of the intracellular domain fragment. Thus, the intracellular domain fragment appeared to derive from the precursor GHR in a proteasome-dependent fashion.

The data in these studies suggest that in cells that lack JAK2 the nascent precursor GHR is a target for endoplasmic reticulum-associated degradation (ERAD) and represent the first example of ERAD-associated cleavage of a cytokine receptor family member that stems from a lack of its cognate JAK (Figure 2). ERAD is a process whereby proteins that fail to fold properly or otherwise fail quality control mechanisms in the ER undergo retrotranslocation and proteasomal degradation in the cytosol [67–69]. JAK2, by virtue of its association with the GHR, rather than via its kinase activity, apparently “chaperones” the dimerized precursor so as to avoid quality control and proceed with efficient processing to mature GHR in the secretory pathway. How does JAK2 exert this chaperone effect? Multiple possibilities exist, including the notion that a receptor region that might otherwise be seen as defective or unfolded to the quality control apparatus is hidden by JAK2 binding. In a similar fashion, JAK2 binding might allosterically alter a GHR site outside of the region that interacts with JAK2 to make that site appear less defective. Unraveling of such possibilities awaits further investigation.

Stabilizing effects on the cell surface levels of respective cytokine receptors have been shown for all members of JAK family including TYK2 (for IFNAR1 [70,71] and thrombopoietin receptor (TpoR) [72]), JAK1 (for oncostatin M receptor [73,74] and IL-9Rα, and IL-2Rβ [72] and Fuchs, et al, unpublished data), JAK2 (for TPOR [72], erythropoietin receptor [75], and GHR [62]) and JAK3 (for the common gamma chain of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptor complexes [76]). Little is known about the mechanisms by which different JAK family members increase the cell surface levels of different receptor chain; models proposed so far include a chaperone-like assistance in receptor folding, maturation and delivery to the cell surface, inhibition of basal endocytosis and/or post-internalization sorting into the late endosomes/lysosomes, as well as protection from proteasome-mediated degradation.

A novel aspect of the work on JAK2 and GHR biosynthesis that extends broadly into the arena of ERAD and proteasome function is the fact that the apparent product of this GHR degradation is a discrete intracellular domain-containing receptor fragment that is stable enough to be detected by immunoblotting. Assuming that the proteasome (rather than a separate proteasome-dependent protease) directly catalyzes GHR ERAD under JAK2-deficient circumstances, such an apparently discrete cleavage is somewhat unusual. Proteasomal degradation is usually thought to involve the threading of target proteins through the proteasome complex's gated substrate channel. Relatively few instances of incomplete or discrete cleavage by the proteasome have been described. Examples include the incomplete cleavage of NF-κB p105 yielding the p50 subunit due to the presence of a "processing stop signal" [77,78] and the endoproteolytic cleavage of certain fusion proteins [79]. Whether either of these mechanisms is in play to explain the ERAD-generated, proteasome-dependent production of the intracellular domain-containing GHR is as yet unknown.

Proteolysis of the GHR: Roles of TACE, γ-secretase, and the Proteasome

Over the last decade, it has become appreciated that GHR, like some other surface receptors, is a target for regulated sequential proteolysis, the first step of which ("α-secretase cleavage") occurs in the proximal extracellular domain stem region 8–9 residues (depending on species) outside the plasma membrane [65,80,81]. This α-secretase cleavage results in loss of full-length GHR, appearance of a cell-associated transmembrane domain (TMD)/intracellular domain (ICD)-containing receptor fragment (the "remnant"), and a soluble GHR extracellular domain (ECD) (called GH binding protein (GHBP), in correspondence with the high affinity GH binding protein that circulates in many species, including humans [82,83]). GHR α-secretase cleavage is constitutive, but can be further induced in various cell types by a protein kinase C activator (the phorbol ester, PMA), platelet-derived growth factor (PDGF), or serum [65,80, 81,84–87]. This cleavage is catalyzed mainly by the extracellular domain of the transmembrane metalloprotease, TACE (tumor necrosis factor-α converting enzyme; ADAM-17) [66,88]. Importantly, inducible α-secretase cleavage likely regulates GH sensitivity; that is, GH-induced signaling is dampened after cells are exposed to stimuli that promote GHR α-secretase cleavage, but not in the presence of metalloprotease inhibitors or if noncleavable receptor mutants are expressed, suggesting that metalloproteolysis modulates GH responsiveness in part by regulating surface GHR levels [65,83,84]. Further, recent *in vivo* experiments indicate that administration of bacterial endotoxin leads to downregulation of hepatic GHR abundance and hepatic insensitivity to GH at least in part by inducing receptor proteolysis, suggesting that this may constitute a physiologically-relevant mechanism of regulation of GH action [89]. Notably, GH itself does not promote GHR α-secretase cleavage; indeed, GH inhibits subsequent GHR proteolysis, apparently by altering GHR conformation, rather than by causing signaling [66].

Recent studies have shown that the α-secretase-generated GHR TMD/ICD remnant is further cleaved by an enzyme activity termed "γ-secretase" within the TMD, which liberates the ICD, a protein termed the "GHR stub" [87]. γ-secretase consists of four molecules, including presenilin, which forms the aspartyl protease core and facilitates a process known as regulated intramembrane proteolysis (RIP) [90]. The GHR stub was detected by immunoblotting most readily when cells were pretreated with either of the proteasome inhibitors, lactacystin or epoxomicin [87], but such treatment had no effect on either the formation or degradation of the remnant from which the stub derives. Thus, the stub is selectively degraded in a proteasome-dependent fashion. Furthermore, the stub can be detected in both the cytosol and the nucleus [87]. The data are consistent with the notion that inducible α-secretase cleavage generates remnant, which is converted to stub by γ-secretase. The stub is labile and can accumulate in the nucleus; proteasome inhibition prevents stub degradation (Figure 3).

The two-step α-/γ-secretase GHR processing is analogous to that seen for amyloid precursor protein, Notch, and others; in those systems, nuclear-localized ICD fragments affect gene expression [90]. It is not yet known whether the GHR stub also regulates gene expression. However, as α-/γ-secretase GHR processing is inhibited, rather than stimulated, by GH, such GHR-mediated gene expression could constitute a unique mechanism by which GH may affect gene expression; that is, by negatively regulating stub formation, rather than by promoting via GHR signaling the activation of STAT5 and STAT5-mediated gene transcription, for example. If such an alternative pathway of GH-modulated gene expression does exist, it will likely be modulated by factors that affect proteasome activity. Future studies will likely address whether the stub indeed associates with chromatin/DNA and, if so, which genes might be regulated by the stub.

Surface GHR Stability: Roles of JAK2 and Proteasome Inhibitors

Once at the cell surface, the GHR could, in principle, achieve several fates. If engaged by GH, signaling is triggered and the receptor undergoes ligand-dependent downregulation (more below). However, in the natural milieu, GH is released from the pituitary gland in a pulsatile fashion such that GH levels are quite low in periods between pulses. Thus, it is critical to understand factors that govern GHR abundance independent of GH. It is believed that mature GHRs are cleared from the cell surface by constitutive or inducible proteolytic shedding and by constitutive downregulation. GHR proteolysis was discussed above. Recent work has revealed important features relating to constitutive downregulation and has, in particular, focused on effects of JAK2 on this process.

Elegant work over the past decade, emanating mainly from the Strous laboratory and performed largely in one cell system (a stably GHR-transfected temperature-sensitive Chinese hamster lung fibroblast that has a thermolabile ubiquitin activating enzyme E1), has suggested that constitutive (and GH-induced) GHR endocytosis requires an intact ubiquitin-proteasome system; an important conclusion drawn was that, although GHR is ubiquitinated, this ubiquitination is not necessary for its endocytosis [91–93] (more below). The so-called ubiquitin-dependent endocytosis (UbE) motif, a conserved ten residue region in the proximal third of the receptor's cytoplasmic domain, is believed to be necessary for efficient GHR endocytosis [94–97]. Furthermore, these studies suggest that even though an intact ubiquitin-proteasome system is required, constitutive GHR endocytosis results in lysosomal, rather than proteasomal, degradation.

As above, JAK2 affects the fate of the cell surface GHR and in cells lacking JAK2, the ratio of mature (cell surface):precursor GHR was substantially reduced in comparison to JAK2-replete cells [25,62]. This finding is partly explained by the chaperone effect of JAK2 during GHR biogenesis [63]. However, notable JAK2-dependent differences in the constitutive fate of mature GHRs are found as well [62]. In the context of a stable reconstitution system, the half-life ($t_{1/2}$) of the receptor was estimated by anti-GHR immunoblotting after 0–4 hours of treatment with cycloheximide (CHX) to inhibit new protein synthesis. The results of such a "CHX chase" assay indicated that the precursor GHR abundance dropped precipitously and to a similar degree with increasing duration of CHX treatment both in cells that did or did not express JAK2. For the mature receptor, however, there was a dramatic effect of JAK2. As measured by this assay, the GHR $t_{1/2}$ increased from roughly one hour in cells that lack JAK2 to roughly four hours in cells expressing JAK2 [62]. Thus, in the absence of GH, it appears that, in addition to its role in shepherding the GHR through the secretory pathway and lessening the degree to which it is targeted for ERAD, JAK2 also extends the receptor's presence at or near the cell surface, presumably by interfering with constitutively active cellular machinery that functions to internalize and downregulate the receptor (Figure 4). Notably, this latter effect has also been detected for another JAK family member, TYK2, with regard to its associated

type I interferon-α receptor (IFNAR1) [71], but not for some other cytokine receptor/JAKs, including the erythropoietin receptor/JAK2 [75] and the oncostatin-M receptor/JAK1 [73].

The degradative pathways operative in conferring the rapid downregulation of mature (presumably cell surface) GHR in cells lacking JAK2 were also studied [62]. In particular, both a proteasome inhibitor (*clasto*-lactacystin *β*-lactone, an active analog of lactacystin) and lysosome inhibitors (ammonium chloride and chloroquine) were tested and inhibition of each pathway similarly prevented loss of the mature GHR upon CHX treatment in the cells that lack JAK2, but no effect was seen in cells that express JAK2 (as the mature GHR $t_{1/2}$ was already quite prolonged in those cells). The results of these studies are consistent with the idea that JAK2 affects surface GHR availability being at the level of constitutive endocytosis and lysosomal degradation and that proteasome inhibition in cells that lack JAK2 blocks the ability of the receptor to enter this pathway. Whether proteasome inhibitor exerts this effect by a more "global" mechanism (eg., by depleting available cellular stores of ubiquitin by blocking turnover of ubiquitinated proteins) vs. specifically impacting the turnover of the GHR or other proteins involved in its constitutive downregulation is as yet unclear. Likewise, further research will be required to definitively discern whether JAK2 functions in this process by inhibiting entry into the endocytic pathway vs. promoting recycling of already endocytosed receptors.

GH-induced GHR Downregulation: Roles of JAK2, GHR Tyrosine Phosphorylation, and Ubiquitination

Like many surface receptors, GHR undergoes important trafficking events in response to binding of its ligand. The net effect is substantial GH-induced receptor downregulation, which serves to limit or alter the receptor's signaling capacity and perhaps thereby further emphasize the physiologic effects of pulsatile GH release from the pituitary gland. Work as early as the 1970s–1980s and since that time suggested that GH-induced GHR downregulation proceeds via clathrin coated pit-mediated endocytosis and lysosomal degradation [91,98–100]. As mentioned above, mutagenesis studies have pointed to a cytoplasmic domain region of the receptor that includes the UbE motif as required for efficient GH-induced GHR endocytosis and downregulation [92,94–97]. While many studies have indicated that GH markedly augments the constitutive rate of GHR downregulation described above, some have suggested only a modest GH-induced increase [93,101]. Similarly, the roles of GH signaling and, in particular, JAK2 activity, in promoting receptor downregulation have been debated. Studies in which chemical kinase inhibitors have been employed suggested that JAK2 kinase activation is required for GH-induced receptor downregulation [102,103]. Like all such inhibitor studies, potential nonspecific effects of the compounds used could hamper interpretation. A different conclusion was drawn by Strous and colleagues in the GHR-transfected temperature-sensitive Chinese hamster lung fibroblast system mentioned above; in this case, a receptor with a mutated Box 1 element (which disrupts GHR-JAK2 association) was downregulated in response to GH similarly to the wild-type GHR, suggesting to the authors that GHR degradation is independent of signal transduction via JAK2 [104].

This question of the role of JAK2 and its activation in GH-induced GHR downregulation compared to constitutive downregulation, was approached recently, again using the reconstitution system described above [105]. In cells that harbor JAK2, GH markedly enhanced GHR degradation; however, in cells that lack JAK2, GH had no effect on receptor degradation (which proceeded at a high level constitutively in the absence of JAK2). Thus, in this system, GH caused receptor downregulation in a JAK2-dependent fashion. Expression of a GHR mutant that lacks the ability to interact with JAK2 resulted in enhanced constitutive receptor downregulation and a loss of GH-induced downregulation. Similarly, the ability of JAK2 to both allow GH-induced receptor loss and to protect GHR from constitutive degradation depended on the presence of an intact (GHR-associating) FERM domain within JAK2. In

distinction, JAK2 mutants lacking the kinase-like and kinase domains did not mediate GH-induced GHR downregulation, despite their ability to protect the receptor from constitutive downregulation. A kinase-deficient JAK2 mutant was also unable to mediate GH-induced GHR downregulation, confirming that kinase activity is required. Notably, a GHR mutant in which all the cytoplasmic tyrosine residues were changed to phenylalanines was also resistant to GH-induced GHR downregulation; this powerful observation indicates that tyrosine phosphorylation of the receptor (rather than JAK2 kinase activation only) is required for GH-induced receptor loss. Interestingly, GH-induced GHR ubiquitination was detected in cells expressing wild-type GHR and JAK2, but not in cells that expressed wild-type GHR and a kinase-deficient JAK2 or in those with wild-type JAK2 and the GHR mutant with all tyrosines changed to phenylalanine, indicating that GH-induced receptor ubiquitination depends on both JAK2 activity and the ability of the receptor to be tyrosine phosphorylated.

Pharmacologic studies in this system showed that a lysosome inhibitor (chloroquine) blocked GH-induced GHR downregulation in cells harboring wild-type GHR and JAK2, consistent with previous findings in other systems that GH ultimately causes its receptor to be degraded in lysosomes. However, as we found for constitutive downregulation of GHR in JAK2-deficient cells (above), a proteasome inhibitor (lactacystin) also blocked GH-induced GHR downregulation. This is consistent with the work of Strous and colleagues (described above) in that intact proteasome activity is needed for effective GH-induced GHR downregulation. But, importantly, in contrast to previous reports, the findings in this system are also consistent with the possibility that GHR ubiquitination itself might play a role in its downregulation.

Tyrosine phosphorylation is essential for downregulation of various cell surface receptors. One of the best studied is the epidermal growth factor receptor (EGFR), which autophosphorylates in response to EGF, allowing association with ubiquitinating machinery, ubiquitination of the receptor itself, and its post-endocytic lysosomal degradation [106]. For GHR, several binding partners have been shown to associate via their SH2 domains with the tyrosine phosphorylated intracellular domain [30,107–111]. One of them, the protein tyrosine phosphatase, SHP-2, may contribute modestly to GH-induced GHR downregulation [108]. More recently, it has been appreciated that the SOCS family protein CIS (cytokine inducible SH2 domain-containing protein), which interacts with tyrosine phosphorylated GHR [109,110] and is likely linked to Cullin5-based E3 ubiquitin ligase complex that can recruit proteins to the proteasome for degradation [112], promotes GH-induced GHR internalization and thus can desensitize GH signaling [113]. Whether CIS regulates GHR downregulation at a level following internalization is not known. Another E3 ubiquitin ligase, β-TrCP (β-transducin repeats-containing protein), may function in the ligand-induced ubiquitination and proteolysis of cytokine receptor family members including interferon-α receptor 1 (IFNAR1), prolactin receptor (PRLR) and erythropoietin receptor [114–118]. Phosphorylation of specific serine residues within the cytoplasmic domain of these receptors allows ligand-induced association with β-TrCP, receptor ubiquitination, and downregulation [115,117]. In the case of IFNAR1 and the PRLR, receptor phosphorylation within the β-TrCP-binding motif required the catalytic activity of the associated JAK (TYK2 for IFNAR1 and JAK2 for PRLR) [119,120] and, like our findings for GHR, ubiquitination of EpoR requires JAK2 activity [121].

β-TrCP has also recently been implicated in GHR downregulation in that knockdown of this molecule retards receptor internalization and increases its steady-state abundance [122]. Furthermore, the GHR cytoplasmic tail and β-TrCP were shown to associate in *in vitro* binding assays, intriguingly via the receptor's UbE motif, rather than via the canonical DSG motif seen for other β-TrCP-binding proteins and also present in the GHR [122]. Whether this novel association and/or β-TrCP itself are the critical determinants of GH-induced receptor downregulation remains unknown in the intact cellular setting. Based on our data above with the GHR that cannot undergo tyrosine phosphorylation, our view is that future studies that

identify proteins that differentially associate with a wild-type vs. tyrosine mutated GHR in response to GH stimulation might shed light on the reasons underlying the defective ligand-induced downregulation of the latter receptor and whether it is due to its inability to be tyrosine phosphorylated, ubiquitinated, or both. This is an interesting area that may yield substantial insight into the mechanisms of downregulation of GHR and, by extension, other cytokine receptor family members.

Mechanistic links between ubiquitination and endocytosis: lessons from GHR and other cytokine/hormone receptors

For the past ten years, an important role of receptor ubiquitination in regulating the rate of their endocytosis has emerged. During this time span, it has been demonstrated that monoubiquitinated cargo receptors are recruited to the proteins containing ubiquitin-binding domains. Accordingly, a prevalent line of thought (supported by experiments utilizing a linear fusion of ubiquitin to the intracellular tail of the receptor) was that such proteins link receptor cargo to the components of endocytic and sorting machinery thereby enabling receptor internalization and post-internalization sorting. Under this scenario, neither polyubiquitination nor the topology of ubiquitin chains nor the site of ubiquitin conjugation should be important for the efficiency of endocytosis [123–128].

However, recent studies on mechanisms that govern internalization of the interferon alpha receptor revealed that site-specific polyubiquitination promotes internalization of this receptor via exposing a linear endocytic motif within the receptor to the interaction with the AP2 adaptin complex which is essential for efficient internalization. The lines of this new paradigm, therefore, suggest that, in the absence of ligand-stimulated ubiquitination, a specific linear motif within the receptor might be masked by receptor-interacting proteins that shield such motif from the AP2 adaptin complex. Upon ligand addition, stimulation of a site- and topology-specific ubiquitination of the intracellular domain of a receptor may result in exposure of the linear motif to adaptin complex either via rearranging the masking protein complex or by changing the conformation of the intracellular tail of a given receptor in a way that enables such interaction. Whereas additional mechanisms leading to an exposure of a linear endocytic motif cannot be ruled out, ubiquitination-stimulated interaction of adaptin with such motif on one hand and clathrin lattices on another hand should promote efficient internalization of a given cytokine/hormone receptor.

TYK2 has recently been identified as a component of this masking complex for interferon alpha receptor (S.Y.F., unpublished data). In cells lacking TYK2 or under the conditions where either TYK2 recruitment is impaired, the receptor undergoes very efficient basal endocytosis that is totally independent of receptor ubiquitination. Other masking determinants that protect various endocytic motifs and, hence, limit basal internalization of other signaling receptors to preserve a physiological density of surface receptors should be eventually identified. In case of GHR, although the identity of linear motifs and masking proteins remain to be determined, the evaluation of this scenario might be useful to understand the counterintuitive results obtained by the Strous group on GHR.

While, in some cases, interacting proteins will been involved (similar to the function of TYK2 for interferon alpha receptor), masking could also be achieved by intramolecular folding of the receptor itself. Thus, the functional role of a given endocytic determinant should always be investigated in the context of the full length receptor. There might be a need to revise the interpretation of data obtained from studies that relied on deletions of the intracellular domains as a strategy for delineating either ubiquitination-related or linear endocytic motifs of signaling receptors. Such deletions might potentially remove not only important positive regulators of endocytosis (such as recognition signal for a ubiquitin ligase or a ubiquitin-acceptor site) but

also docking sequences or surfaces involved in recruitment of masking proteins. In support of this hypothesis, the ubiquitin-independent role of di-leucine linear endocytic motifs that emerge in a truncated GHR has been reported [95].

As per the role of either ubiquitination or linear motifs or both in clathrin-mediated internalization of cell surface receptors, GHR will likely fall into the group for which cooperation between ubiquitination and linear endocytic motifs might serve as a major mechanism for promoting the internalization of these receptors. While a candidate linear motif that might be important for such interaction within the intracellular domain of GHR has been revealed [129], and GHR has been the first mammalian receptor for which overall role of ubiquitination for its endocytosis and degradation has been demonstrated [95] and further delineated by an elegant work of the Strous group [19,91–94,96,104,129–136], the role of GHR ubiquitination per se (as opposed to ubiquitination of the components of endocytic machinery) in internalization and degradation of GHR remains controversial. The bulk of available data strongly suggest a logical connection between GHR ubiquitination followed by its endocytosis followed by the proteolytic turnover in the lysosomes. Furthermore, similarly to IFNAR1 [114,137] and PRLR [116,120], β-TrCP-based E3 ubiquitin ligase interaction (via a non-canonical UbE domain instead of the canonical phosphodegron) and Cullin1-dependent E3 ligase activity governs the endocytosis and degradation of GHR [95]. However, the latter report also suggests that ubiquitination of GHR itself does not largely affect the rate of GHR endocytosis. This conclusion is based on the observation that mutation of all of lysine residues within the cytoplasmic tail of a truncated rabbit GHR into arginines did not affect its endocytosis [96]. However, while ubiquitination of truncated receptor (whose linear motifs might be already exposed to interacting with AP2) for its internalization might be dispensable, the same is not necessarily true for the full length receptor. Work of the Pellegrini group has demonstrated that IFNAR1 mutants lacking Tyk2-binding sites (but retaining phospho-degron and, therefore, ubiquitination) undergo very efficient endocytosis [71]. It remains to be seen whether abrogation of the full length GHR ubiquitination by means that do not involve its extensive mutagenesis, which might activate alternative or downstream internalization pathways, will not be essential for its efficient endocytosis. Another possibility is that, in the absence of lysines, ubiquitination might occur on the cysteine residues. Furthermore, such ubiquitination can stimulate endocytosis as has been demonstrated for the major histocompatibility complex class I molecules decorated by viral E3 ubiquitin ligases [138]. In all, given these relatively recent developments, the issue of the role of GHR ubiquitination in its internalization, post-internalization sorting and lysosomal degradation might worth being revisited.

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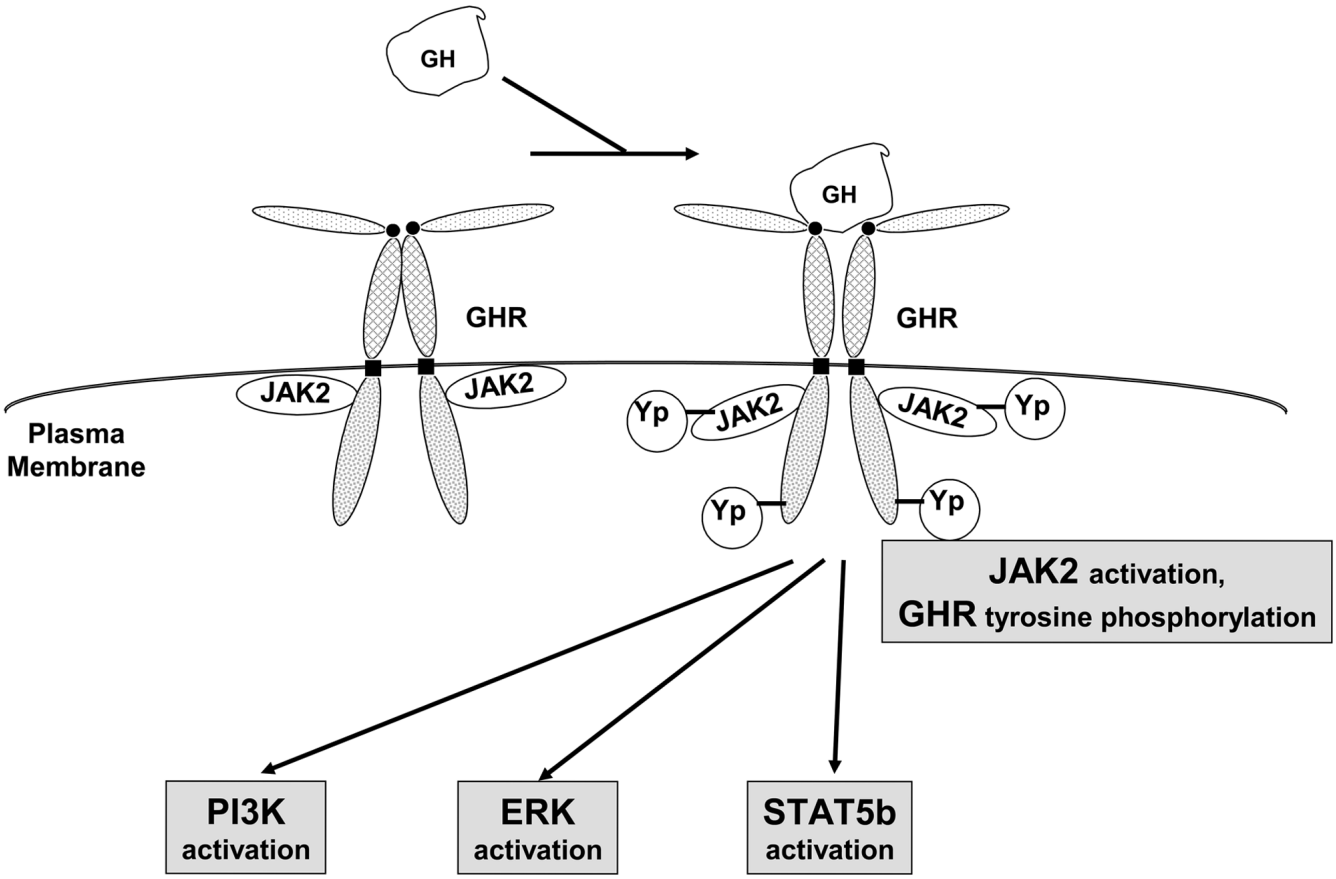

Figure 1.
GH signaling pathways. GH binds to the cell surface dimerized GHR, causing JAK2 activation and activation of downstream pathways, including STAT5, PI3-kinase, and ERK pathways.

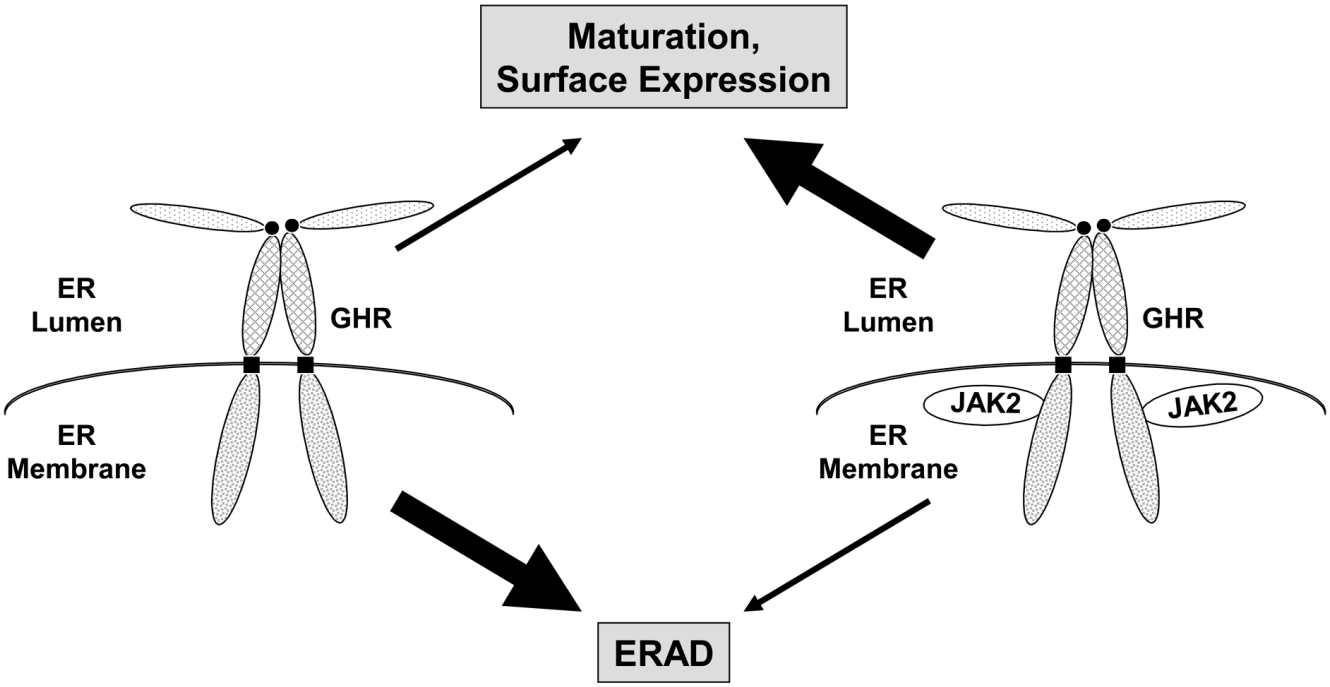

Figure 2.
JAK2 association affects endoplasmic reticulum to cell surface GHR trafficking. In cells harboring GHR and JAK2 molecules that can associate, GHR moves from the ER to the Golgi, matures, and reaches the cell surface efficiently. In cells lacking JAK2 or with GHR molecules that cannot associate with JAK2, GHR undergoes endoplasmic reticulum-associated degradation (ERAD) and inefficiently matures and trafficks to the cell surface.

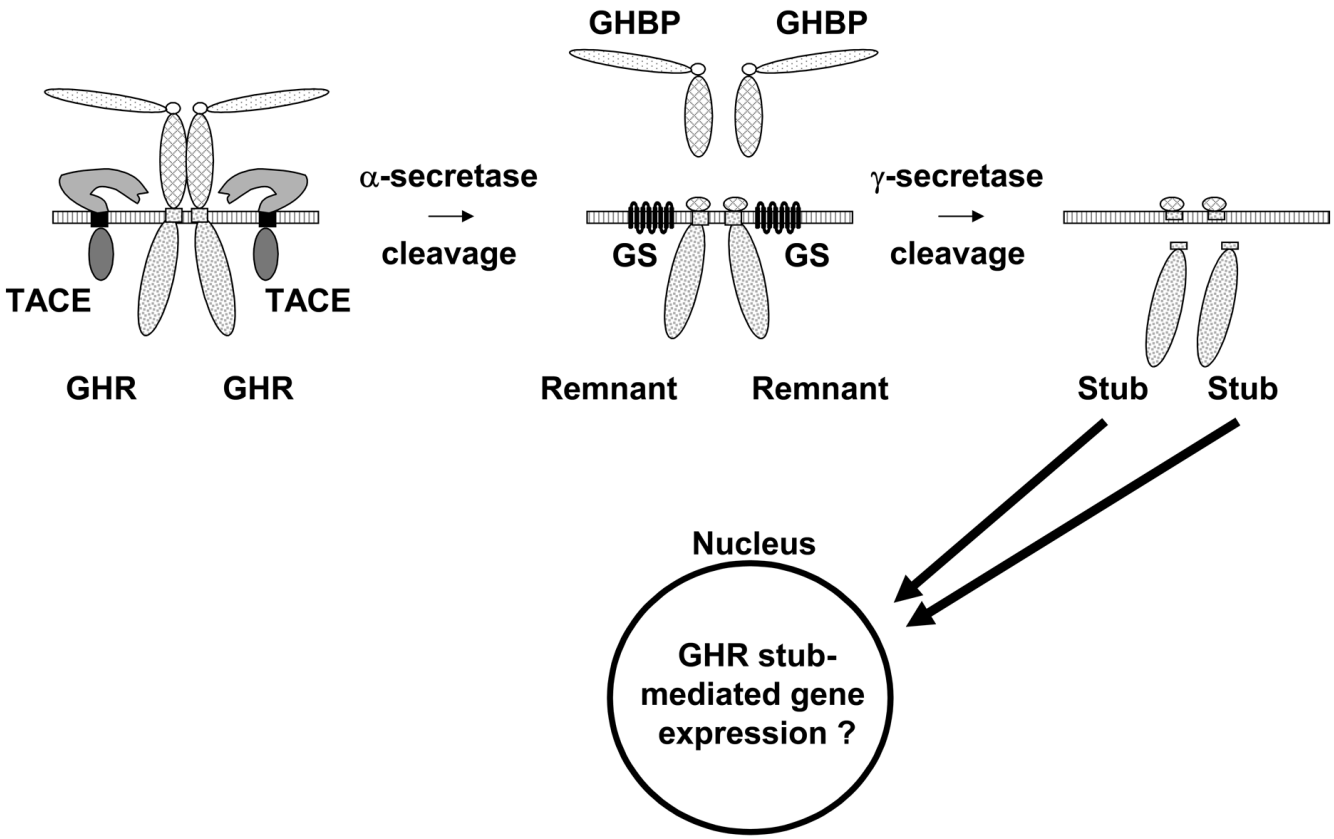

Figure 3.
GHR undergoes sequential TACE and gamma-secretase cleavage. Surface GHR undergoes constitutive and inducible cleavage in the extracellular domain stem region by TACE in a process called “alpha-secretase” cleavage. This yields the shed GHBP and the GHR remnant. Remnant is then cleaved by gamma-secretase within the membrane to yield the GHR stub (soluble intracellular domain), which localizes to the nucleus, where it may affect gene expression.

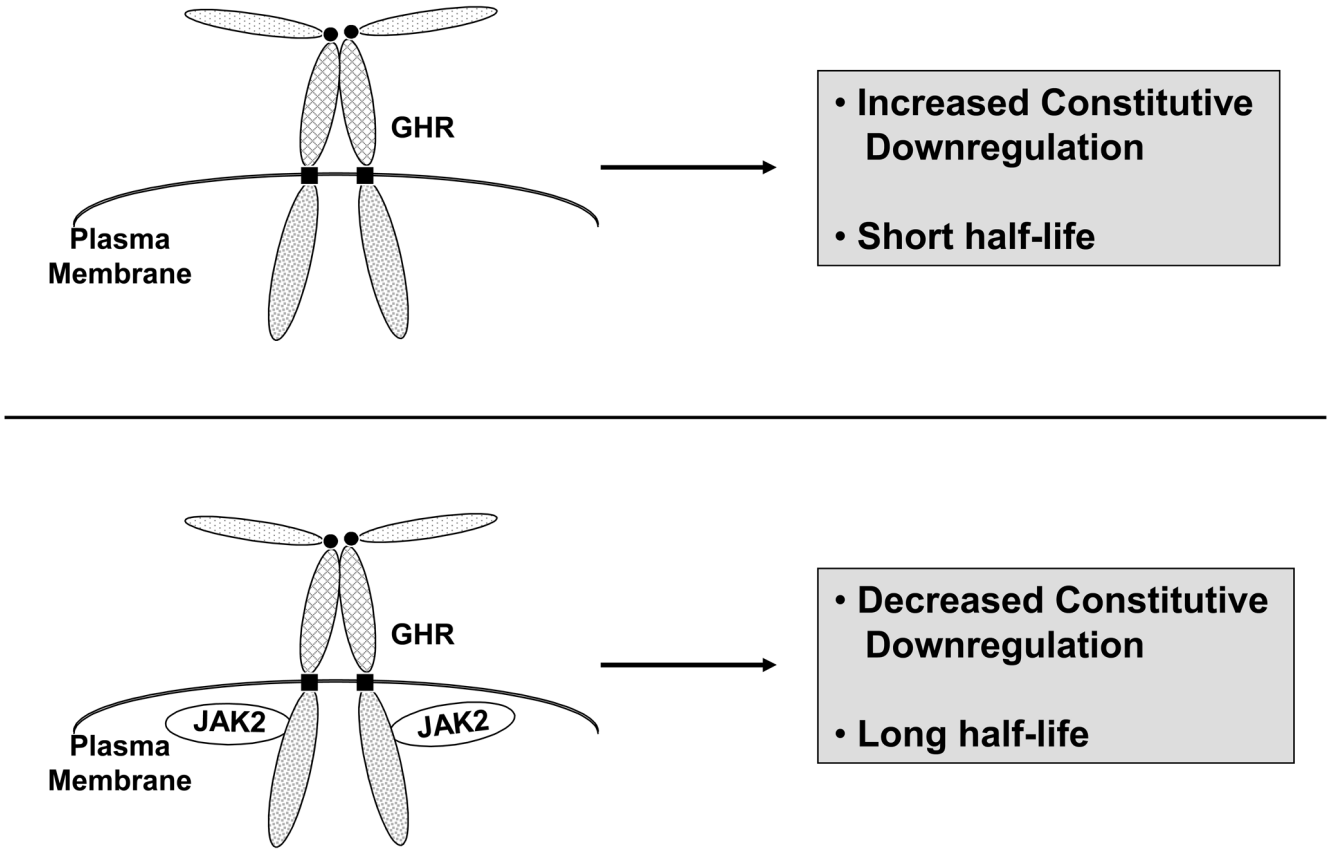

Figure 4.
JAK2 association affects the constitutive (GH-independent) fate of surface GHR. In cells harboring GHR and JAK2 molecules that can associate, surface GHR is downregulated at a low constitutive rate and its half-life is long. In cells that lack JAK2 or have GHR and JAK2 molecules that cannot associate, GHR undergoes enhanced constitutive downregulation and exhibits a short half-life.