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DETERMINANTS OF GROWTH HORMONE RECEPTOR DOWNREGULATION

by

LUQIN DENG

STUART J. FRANK, CHAIR
JIM COLLAWN
JOSEPH L. MESSINA
CHENBEI CHANG
WEEI-CHIN LIN

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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2008

DETERMINANTS OF GROWTH HORMONE RECEPTOR DOWNREGULATION

LUQIN DENG

CELL BIOLOGY

ABSTRACT

Growth hormone (GH), a 22 kD polypeptide primarily produced in the anterior pituitary gland, is a key regulator of postnatal growth and affects carbohydrate, protein and lipid metabolism. Growth hormone receptor (GHR) is a single membrane-spanning type Ⅰ glycoprotein of the cytokine receptor family and is initially synthesized as a precursor and undergoes carbohydrate processing during transport through the Golgi. It has no intrinsic kinase activity. One molecule of GH binding to predimerized GHR on the cell surface induces the receptor conformational change activating the cytoplasmic domain-associated tyrosine kinase, Janus Kinase 2 (JAK2), which phosphorylates GHR, followed by the activation of downstream pathways, including MAPK, STATs and PI3K. Thus, GH sensitivity is largely determined by cell surface GHR abundance. Previous studies have demonstrated that JAK2, in addition to its role in GH signaling, enhanced GHR maturation, mature GHR stability and surface availability. By reconsititution of γ2A-GHR or γ2A-JAK2 cells with mutant JAK2 or mutant GHR respectively, we found that the membrane proximal region of GHR called Box1 element and intact N-terminus of JAK2 are necessary and sufficient for the JAK2-dependent stabilization of the GHR in the absence of its ligand. In contrast, neither the kinase domain nor the kinase-like domain of JAK2 is required for this effect. Furthermore, we investigated the effect of JAK2 on GH-induced GHR loss. Our data suggested that in the presence of JAK2, GH treatment markedly enhances GHR degradation in a dose-dependent manner. However, in

contrast to constitutive GHR loss, GH-induced receptor downregulation relies not only on GHR-JAK2 association but also on JAK2 kinase activity and GHR tyrosine(s). Tyrosine mutation of GHR diminished GH-induced GHR internalization. Mutation of the serine residues in DSGRTS motif of GHR did not impair GH-induced GHR degradation. Both lactacystin (a proteasome inhibitor) and chloroquine (a lysosome inhibitor) blocked GH-induced GHR loss. Further experiments also indicate that, like downregulation, JAK2 kinase activity and GHR tyrosine(s) are also critical for GH-dependent ubiquitination of GHR.

DEDICATION

To my parents, for their love and understanding; to my little angel, Tina; and most importantly to my husband, Fangming, for his love and support.

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First, I want to thank my mentor, Dr. Stuart Frank. I am where I am today because of his patience, encouragement and guidance throughout my graduate studies under his tutelage. There are not enough words to express my appreciation and gratitude for him. I would especially like to thank Jing Jiang for her excellent organization for our lab and Xiangdong Wang for his patience and help during last five years. Kimberly Loesch, a nice colleague and I appreciate her help in reading my dissertation. Thanks to Kai He for his guidance, too. I also appreciate the help from other people in the lab, including Kai He, Yao Huang, Ning Yang, Jon Cowan, Yue Zhang, Liang Liu, Xin Li, Yunjun Gan, Jie Xu. I will remember the wonderful time we had. Last, I also want to thank my committee for their patience, support and helpful suggestions for my research.

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PART 2

INTRODUCTION

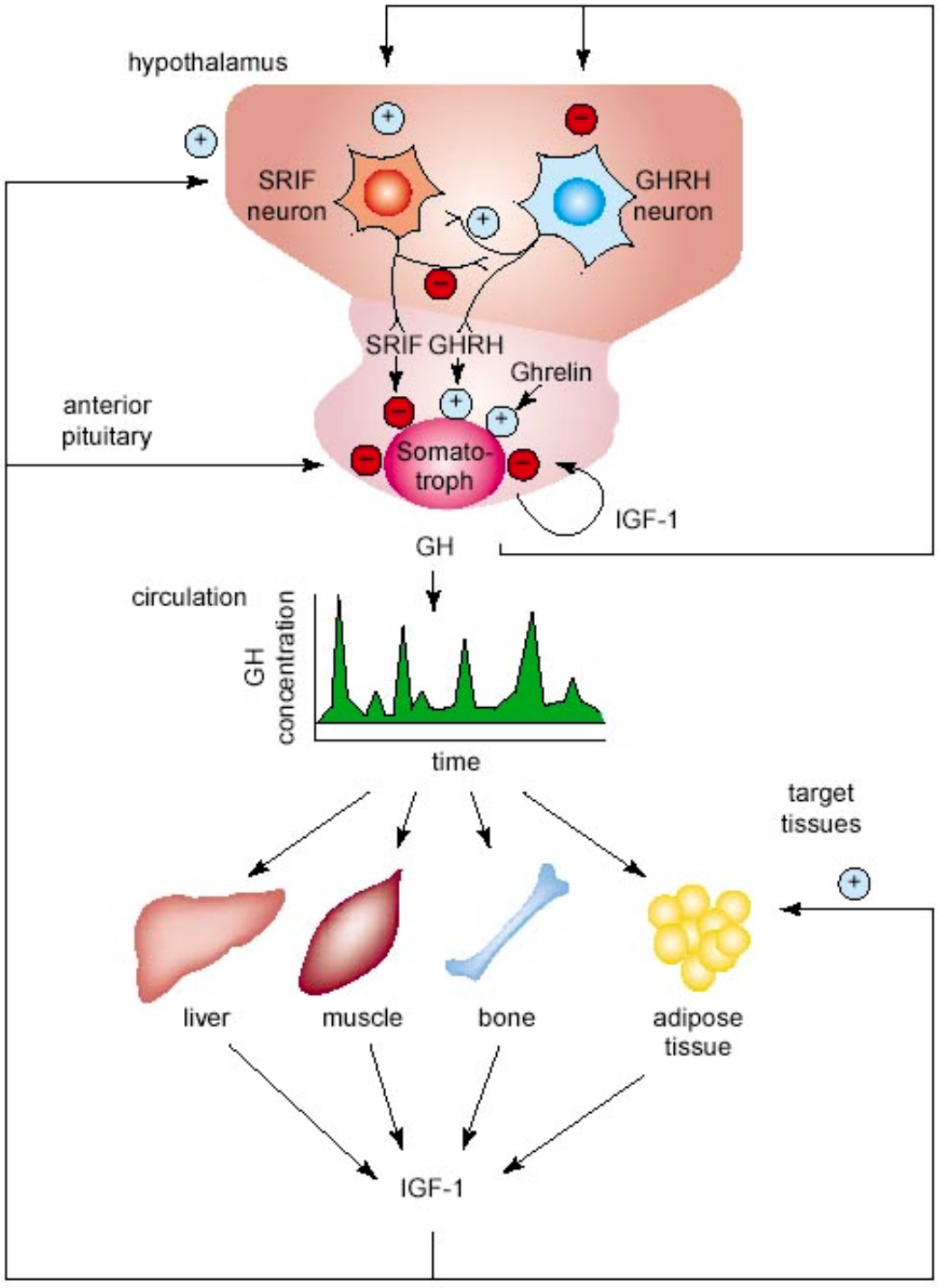
Growth Hormone Physiology

Growth hormone (GH) is a member of a hormone family that includes prolactins and lactogens (1) and it is primarily synthesized in the anterior pituitary gland. The production of GH has also been found in brain (other than pituitary gland), pre-implantation embryo, placenta, extrapituitary tumors, mammary and lymphoid tissues at relatively low levels (2). The secretion of GH from the pituitary gland is regulated through the functional interplay of two hypothalamic hypophysiotropic hormones, GH-releasing hormone (GHRH) and somatostatin, which exert stimulatory and inhibitory effects on circulating GH levels respectively (3-6) (Figure 1). In addition, other factors, including free fatty acids, leptin, neuropeptide Y and ghrelin, can coordinate the metabolic status of the organism with GH secretion to influence GH release (7-10). GH is secreted from pituitary in a pulsatile pattern consisting of intermittent peaks with prolonged interpulse troughs (11, 12). This episodic fashion of GH secretion is more efficient in improving growth compared to constant infusion in GH-deficient animals (13).

As a major regulator of postnatal growth in vertebrates, GH has been shown to have direct effects on bone growth (14, 15); however, the growth-promoting effect of GH is largely fulfilled through up-regulating hepatic gene expression and production

Figure 1. Schematic representation of the regulation of growth hormone (GH) secretion.

Somatostatin (SRIF) and GH releasing hormone (GHRH) hypotharamic neurons control each other by direct synaptic connections. SRIF and GHRH are released from the median eminence of the hypothalamus and reach somatotorophic cells of the pituitary via portal vessels and inhibit or stimulate GH secretion, respectively. GH regulates its own secretion either by stimulating or inhibiting the secretion of SRIF and GHRH. GH inhibits GHRH mRNA synthesis and GHRH release in the hypothalamus, and stimulates mRNA synthesis of SRIF and SRIF release. GH also stimulates insulin-like growth factor (IGF-1) secretion from somatotrophic cells (ultra short feedback loop). GH is released into the circulation as secretory spikes. The circulating GH then binds to GHRs on target tissues including liver, muscle, bone and adipose. GH promotes paracrine or endocrine secretion of IGF-1. that, in turn with GH, stimulates tissue proliferation. IGF-1 produced in the target tissues, in the long feedback loop scheme, will inhibit GH secretion either directly at the somatotrophs or indirectly by stimulation of SRIF release. GHRP, ghrelin, is secreted from the stomach and is believed to act on somatotrophic cells. Adopted from Ref. (6).


hypothalamus
SRIF neuron
GHRH neuron
SRIF GHRH
Ghrelin
anterior pituitary
Somato-troph
IGF-1
GH
circulation
GH concentration
time
target tissues
liver
muscle
bone
adipose tissue
IGF-1

of insulin-like growth factor-1 (IGF-1) (16-18). Acting in a negative feedback loop, increased IGF-1 levels can also inhibit GH seceretion from pituitary.

Both excessive and deficient GH secretion can lead to clinical syndromes. Patients with acromegaly or gigantism are indicative of GH excess while GH-deficient patients are short in stature and have abnormal skeletal growth (19). In addition to its growth promoting effects, GH is also involved in lipid, carbohydrate and protein metabolism (20-23).

Growth Hormone

The gene encoding human growth hormone is located on chromosome 17 and contains five exons and four introns. GH polypeptide contains 191 amino acids and possesses a molecular mass of approximately 22,000 daltons. It is comprisesd of four antiparallel α-helical bundles (24). In some species, alternative splicing of GH mRNA, which deletes 15 amino acids, generates an additional isoform of 20,000 daltons variant. Both forms of growth hormone have similar biological functions (25).

Growth Hormone Receptor

The cognate receptor of growth hormone, growth hormone receptor (GHR), belongs to cytokine receptor superfamily based on limited homology in the extracellular domain, including two pairs of cysteines and a WSXWS motif, and in the cytoplasmic domain one or two proline-rich motifs (26). This superfamily presently consists of

receptors for growth hormone, prolactin and leptin, for most of the interleukins (ILs) (ILs 2-7, 9-13), interferons α/β and γ (IFN), erythropoietin, thrombopoietin, granulocyte colony-stimulating factor (G-CSF), oncostatin M, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNF), granulocyte macrophage colony-stimulating factor (GM-CSF), and cardiotropin (26).

GHR is a widely distributed single membrane-spanning type I glycoprotein. It is expressed in many tissues, such as, bone (27), muscle (28), adipose (29), brain (30, 31) and immune tissues (32), but is most abundantly in liver (33). The receptor was initially cloned from rabbit and human liver cDNA libraries (34). The importance of GHR in regulation of growth is illustrated by the detection of deletions and point mutations in the gene encoding GHR in individuals with Laron dwarfism (35-37). The function of growth hormone receptor is also illustrated by GHR knockout (-/-) mice. $GHR^{-/-}$ mice display a dwarf phenotype, have low levels of IGF-Ⅰ, very low levels of insulin, are extremely insulin-sensitive and have an extended lifespan (1, 38). The gene encoding the human GHR is located on chromosome 5p13-p12 and contains nine coding exons. The sizes of full-length GH receptor are in the range of 600 amino acids in different species. The full-length human and rabbit GHR (hGHR and rbGHR, respectively) consist of 638 amino acids, of which, the first 18 N-terminal amino acids is the signal sequence. Functionally active full-length mature hGHR and rbGHR is comprised of 620 amino acids, including 246 residues of ligand-binding extracellular domain, 24

amino acids (residues from 247 to 270) of transmembrane domain and a 350 residue cytoplasmic domain (Figure 2A).

The GHR^{ECD} (amino acids 1-246 of hGHR and rbGHR) contains six (three pairs) conserved cysteine residues that engage in the formation of intramolecular disulfide linkages and a WSXWS-like motif (YGEFS) which is common to members of the Class I cytokine receptors and likely crucial to maintain the structural integrity of the extracellular domain of the receptor. Crystallography studies found that GH binds to the ECD to form a trimetric complex of two receptors and a single molecule of hormone (39) (Figure 2C). In the complex, the two binding sites of GH (site 1 and site 2) engage the receptor differently. The GHR^{ICD} contains elements responsible for GH signal transduction, receptor internalization and downregulation (34, 40). Important features in GHR^{ICD} are: membrane proximal proline-rich sequence called Box1, which is critical for association with the Janus family tyrosine kinase, JAK2 (41); six conserved tyrosine residues (Y314, Y469, Y516, Y548, Y577 and Y609 in hGHR; Y332, Y487, Y534, Y566, Y595, Y627 in porcine GHR), which are phosphorylated by JAK2 when GH binds to GHR and are thus important in signal transduction (42, 43); highly conserved sequence DSWVEFIELD called ubiquitin-dependent endocytosis (UbE) motif which is believed to mediate GH-induced ubiquitination and required for GH-induced receptor internalization and degradation (44-49); DSGXXS domain, a β-TrCP E3 ligase binding consensus motif, function of which is yet to be determined (50).

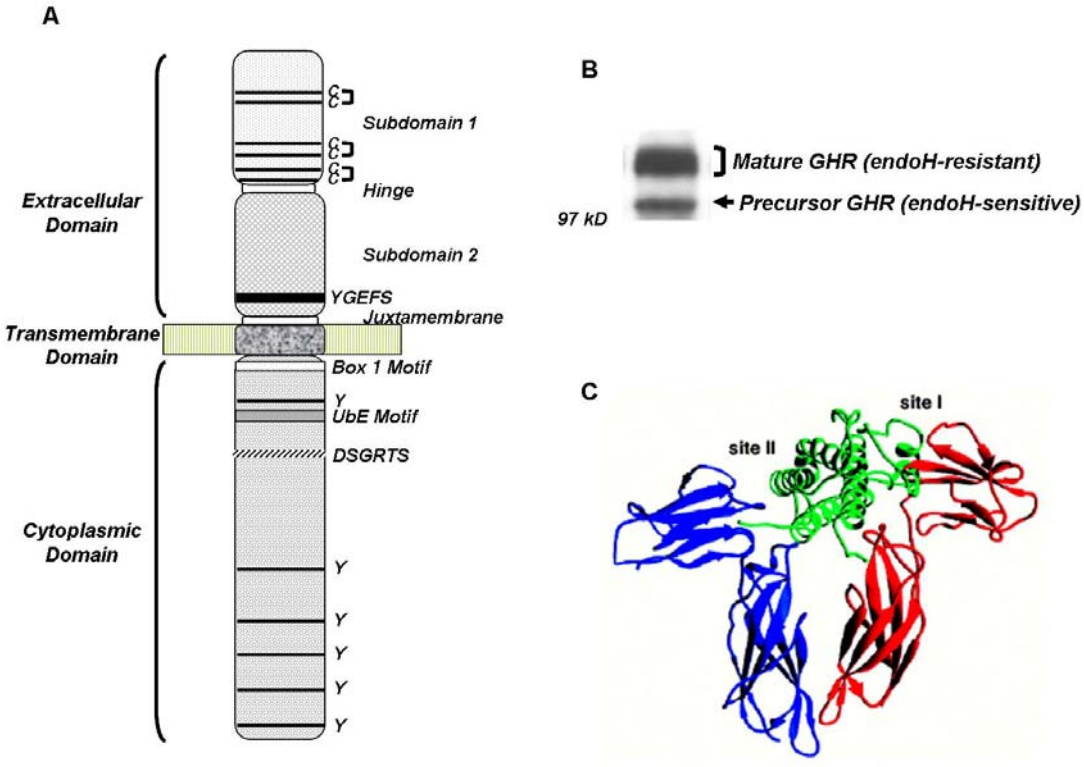


Figure 2, GHR protein structure and the GH:GHR complex.

A, Diagram of the human GHR protein structure. The extracellular, transmembrane, and cytoplasmic domains are indicated. The extracellular subdomains 1 and 2 are shown, as well as the hinge and juxtamembrane regions. The positions of the intrachain cysteine-mediated disulfide linkages present in the extracellular subdomain 1 in GHRs of all species are indicated by pairs of joined C residues. YGEFS is the WSXWS-like equivalent of the GHR. The Box 1, DSGRTS, UbE motifs and the conserved tyrosine residues (Y) in the cytoplasmic domain are shown. B, two forms of GHR in SDS-PAGE: mature GHR which is endoH-resistant and precursor GHR which is endoH-sensitive. C, The structure GH bound to the soluble GHR extracellular domain as determined by crystallography. GH binds to similar contact points on the GHR via distinct sites, site 1 and 2, resulting in the GH:$GHBP_2$ complex shown. A is adapted from Ref. (96). C is based on information in Ref. (39).

The GHR is synthesized as a non-glycosylated protein and dimerizes in the ER (51). In the process of transport through the Golgi, the GHR acquires carbohydrate. However, mature N-glycosylated GHR is achieved by the removal of high-mannose sugars that are added in the ER during the transition from the early to late Golgi (52). Thus, two forms of GHR are detected by SDS-PAGE: mature GHR which is endoglycosidase H (endoH)-resistent and more rapidly migrating high-mannose GHR precursor which is sensitive to deglycosidation by endo H (53) (Figure 2B). The cell surface is populated by the mature form of the receptor.

Janus Kinase 2

The cytokine receptor superfamily members lack intrinsic tyrosine kinase activity and therefore signal via non-receptor tyrosine kinases of the Janus kinase (JAK) family. The family was named after the two-faced Roman god of gates and doorways and exists in numerous vertebrate species (54). There are presently four identified mammalian members: JAK1, JAK2, JAK3 and Tyk2, which range from 120 to 140 kDa (26). JAK1, JAK2, and Tyk2 are ubiquitously expressed while JAK3 is primarily detected in hematopoietic system (54, 55). The three-dimensional structure of the JAKs is still unknown. Based on sequence similarities between JAK family members, seven JAK homology (JH) domains (JH1-JH7) were revealed (56) (Figure 3). The primary domain structure of JAKs are roughly divided into an amino-terminal region (N), adjacent

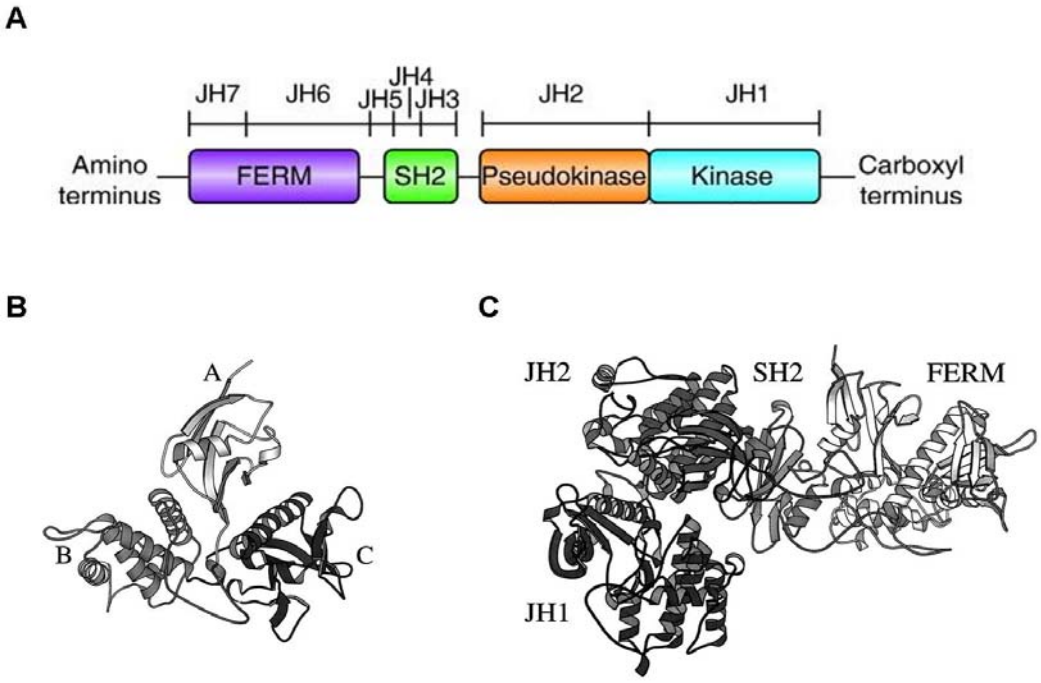


Figure 3. Schematic diagram of JAK structure and computationally predicted structure of JAK2 and its FERM domain.

A, A schematic representation of the primary structure of Janus kinase 2 (JAK2), which is made up of FERM domain and SH2-like domain in the N terminal half and kinase-like and kinase domain in the C terminal part of JAK2. B, Three-lobed (A, B and C) structure of JAK2 FERM domain. C, Computationally predicted JAK2 structure. A is adapted from Ref. (56). B and C are adapted from Ref. (65).

catalytically inactive kinase-like (KL) domain and carboxyl-terminal tyrosine kinase (TK) domain (54, 56). The N terminal region of the JAKs, spanning half of the protein and comprising JH3-JH7 regions, is involved in binding to cytokine receptors (57-62). Part (JH6-JH7) of the N terminal region shares significant sequence similarity with the so called four-point-one, ezrin, radixin and moesin (FERM) domains (63, 64), which by computer modeling adopts a three-lobed structure (65). The regions of JH3-JH4 comprise an SH2-like domain, while a pseudokinase (kinase-like) domain is represented by JH2 region, which has a kinase domain fold but possesses no crucial residues for catalytic activity and for nucleotide binding (55). The JH1 domain at the C-terminus is a classical kinase domain and most closely related to the kinase domains of epidermal growth factor family of receptor tyrosine kinases (56). Like other tyrosine kinases, maximal stimulation of JAK activity is achieved upon the phosphorylation of tyrosine located in a putative activation or A loop in the TK domain (66).

JAK family members are involved in a large number of signaling transduction pathways (see Table 1) and their essential and specific functions were illustrated by JAK knockout mice (54, 56). $JAK1^{-/-}$ mice are small and die perinatally probably due to the neurological defects that prevent them from suckling (67). JAK2 deficiency results in embryonic lethality owing to a defect of erythropoiesis (68, 69). Mice are viable and fertile when JAK3 is knocked out, however, they exhibit severe combined immunodeficiency (SCID) (70-72). $Tyk2^{-/-}$ mice have defective responses to

lipopolysaccharide (LPS), are resistant to collagen-induced arthritis and are susceptible to parasite infection (56, 73).

Table 1. Activation of JAK kinase by some cytokines and growth factors	
JAK kinase	Ligand/Receptor
JAK1	Families of receptor with the shared subunits γc (including interleukin (IL)-2, IL-4, Il-7, IL-9, IL-15 and IL-21) or pg130 (including IL-6, IL-11, oncostatin M, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNF), granulocyte colony-stimulating factor (G-CSF)); IFNs
JAK2	Hormone-like cytokines (such as growth hormone (GH), prolactin (PRL), erythropoietin (EPO), thrombopoietin (TPO)), IL-3, interferon-γ (IFN-γ), family of receptors with the shared subunit gp130 (see above))
JAK3	Family of receptor with the shared subunit γc (shown above)
Tyk2	IL-12, bacterial lipopolysaccharide (PLS)

Growth Hormone Signaling

Historically, it was believed that GH signaling begins with the sequential binding of each of two distinct sites (site 1 and site 2) on GH to two monomers of GHR (39, 74). However, recent studies indicates that the GHR dimer pre-exists in an unliganded state (51). Growth hormone binding to dimerized GH receptors on the cell surface induces their conformational change, resulting in JAK2 activation (40, 75-77). However, the mechanism(s) by which GH binding leads to JAK2 activation is still elusive. Presumably, the conformational change of GHR brings the cytoplasmic JAK2 kinases

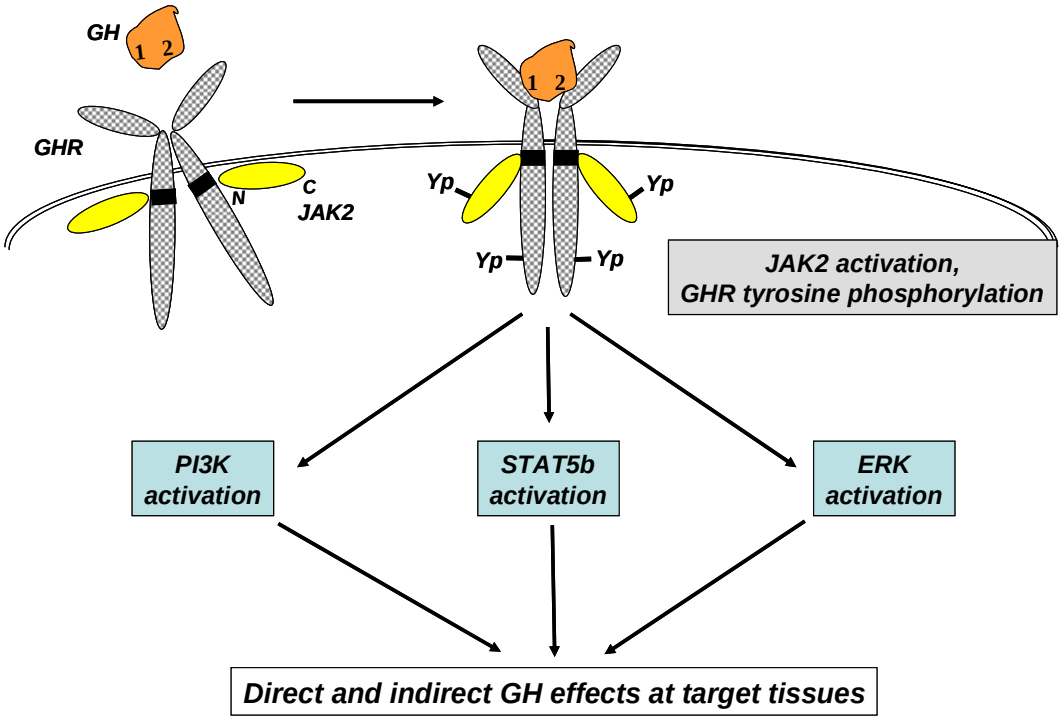


Figure 4, Schematic GHR signaling pathways.

Binding of GH, via site 1 and site 2, to the predimerized GHR on the cell surface induces a conformational change in the receptor activating the cytoplasmic associated tyrosine kinase, JAK2, which initiates the STAT transcription factors, MAP kinase, and PI3 kinase pathways. Tyrosine phosphorylation is indicated by Yp. Adapted from Ref**.** (96)**.**

into proximity with one another, enabling activation of JAK2 by trans- and auto-phosphorylation (78-80). Activated JAK2 subsequently phosphorylates the conserved tyrosine residues within the GHR cytoplasmic domain, thus initiating the GH signaling pathways (81, 82). These phosphotyrosines are important docking sites for scaffolding proteins with SH2 (src homology 2) domains or PTB (phosphotyrosine binding) domain. By the virtue of the docking sites, the signal transducer and activator of transcription 5 (STAT-5) binds to GHR and undergoes tyrosine phosphorylation, dimerize via phosphotyrosine–SH2 interactions and translocate into nucleus and promote transcription of genes including IGF-1, which interact with IGF-1 receptors (IGF-1R) in tissues that respond by growing. GH stimulation also activates the MAP kinase pathway resulting in the transcription of *c-fos* and cell proliferation, and the PI-3 kinase pathway via phosphorylation of insulin receptor substrates (IRS-1 and IRS-2) (Figure 4) (40, 83).

Growth Hormone Receptor Cell Surface Availability

GH signaling is initiated by GH interacting with GHR on the cell surface. Thus, understanding factors that regulate GHR surface abundance is critical for understanding GH action. 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 (84-86). After synthesis in endoplasmic reticulum (ER), GHR abundance is regulated at several levels,

including trafficking to the cell surface, metalloprotease-mediated GHR processing, constitutive and GH-induced GHR downregulation via endocytosis.

After synthesis in endoplasmic reticulum (ER), the existence of properly folded proteins from ER is governed by the ER quality control system. ER-associated degradation (ERAD) is part of the quality control mechanism (87-89). Studies comparing JAK2-deficient vs. JAK2-replete cells indicate that ERAD of GHR is greatly enhanced in the absence of JAK2, suggesting that JAK2 facilitates proper GHR folding during biosynthesis (90). JAK2, a potential chaperone, associates with the GHR in the ER to avoid the proteasomal degradation and enables GHR maturation and proceed efficiently to the cell surface (53, 91).

After reaching the cell surface, the mature GHR proteins may be subject to proteolysis, resulting in shedding of the extracellular domain, or undergo endocytosis via clathrin-coated pits followed by degradation in lysosome (48, 49, 92-95). Inducible proteolytic shedding of the membrane-bound form of GHR produces a soluble form of GHR extracellular domain, the GH-binding protein (GHBP) (96). Tumor necrosis factor (TNFα)-converting enzyme (TACE or ADAM-17) has been identified as a sheddase (97, 98) which cleaves GHR at the site eight residues from the membrane in the proximal extracellular domain stem region (99, 100). This metalloproteolytic cleavage was greatly enhanced in cells expressing JAK2, an effect dependent on GHR-JAK2 interaction (101). GHR is also subject to subsequent γ-secretase cleavage (102). The sequential proteolysis by metalloprotease and γ-secretase activities also regulates GH

signaling (102, 103). GHR endocytosis requires an intact ubiquitin-proteosome system and the ubiquitin-dependent endocytosis (UbE) motif, which consists of the conserved amino acid sequence DSWVEFIELD in the membrane proximal region of GHR cytoplasmic domain and serves as the binding site for the ubiquitin conjugation system (44, 45, 48, 104). Mutations in this domain affect both receptor ubiquitination and receptor internalization (44-46, 92). In endosomes, internalized proteins are either recycled back to plasma membrane or targeted to the lysosome for degradation. For the GHR, no evidence for a significant recycling to the cell surface has been obtained (48, 105).

Recent studies suggest that JAKs, in addition to their roles in signaling, may regulate plasma membrane expression and trafficking of their cognate receptors. JAK1 co-expression increased the fully glycosylated form of the oncostatin M receptor (OSMR) and enhanced its localization on the cell surface. The association of N-terminal region of JAK1 with the membrane proximal region of the receptor, but not its kinase activity, is required for this effect (106, 107). The erythropoietin receptor (EpoR) is a protein with a short half life. It is degraded via multiple pathways and less than 1% of total cellular EpoR are found on the cell surface. The vast majority of transfected erythropoietin receptor (EpoR) is retained in the ER and undergoes degradation without reaching the Golgi apparatus (108). JAK2 expression increases the Golgi-processed form of EpoR by association with immature EpoR through its N-terminal region (109). In contrast to the α subunit, the gamma(c) subunit (γ_c) of the interleukin-2 receptor complex poorly

localizes to the plasma membrane. Although JAK3 is not required for mediating or potentiating the surface expression of γ_c and plasma membrane turnover of γ_c is also independent of JAK3, over-expression of JAK3 increases the accumulation of γ_c at the plasma membrane. Full-length JAK3 is required for this effect although its kinase activity is not necessary (110). Interferon-alpha receptor 1 subunit (IFNAR1) is weakly expressed on the cell surface. Co-expression of Tyk2 slows down IFNAR1 degradation by partially inhibiting its endocytosis and therefore greatly enhances the cell surface expression of IFNAR1 (111). The interaction with JAK2 or Tyk2 promotes thrombopoietin receptor (TpoR) cell surface localization and stabilizes mature TpoR by protecting it from proteasome degradation (112). The sequences encompassing box1 and box2 regions of the receptor cytoplasmic domain and an intact JAK2 or Tyk2 FERM domain are required for these effects (112). Collectively, these data indicate that JAKs may fulfill important roles other than triggering downstream signaling by cytokine receptors, although the mechanisms underlying the impacts of JAKs vary among different receptors. In some instances, the primary mechanism appears to be a chaperone effect that allows efficient surface expression. In others, protection from degradation of the mature surface receptor seems most important. For the GHR, JAK2 can chaperone the receptor and avoid ERAD probably by promoting efficient folding of GHR (90) and enhancing its maturation (91). JAK2 also stabilizes the GHR which increases the accumulation of GHR on the cell surface (53). However, how JAK2 affects GHR surface presentation is yet to be determined.

In addition to activating signaling pathways, ligand binding can also regulate the fate of the receptor. Much research has focused on epidermal growth factor receptor (EGFR) downregulation, which requires multiple signals, including intrinsic kinase activity, endocytic sequence motifs located in the cytoplasmic domain of the receptor, receptor ubiquitination as well as numerous signals in endosomes that direct receptors for lysosomal degradation (113). Ligand-induced GHR downregulation has been reported; however, the degree to which GH enhances constitutive GHR degradation is relatively unclear. Following GH treatment, GH binding sites were reduced at the cell surface (114) while only subtle decrease of the newly synthesized GHR under GH stimulation was observed by Strous et al (115). The role of JAK2 in GH-induced GHR downregulation is also uncertain. Although previous studies suggested that blockade of GH-induced JAK2 activation prevents receptor degradation by using chemical kinase inhibitors such as the tyrphostin AG490 and staurosporine (114, 116), the non-specific property of these inhibitors makes it hard to conclude that JAK2 is involved in the GH-induced receptor downregulation. Further, it has been reported that a mutant GHR with mutations in Box1 element underwent GH-induced downregulation similar to a wild-type GHR although this mutant can not interact with JAK2 (117). This indicates GHR degradation may be independent of signal transduction via JAK2. By studying a truncated GHR, Strous and colleagues concluded that ubiquitination of the GHR itself is not required for GH-induced endocytosis and degradation, although an intact

ubiquitin-conjugating system was required (44). Whether tyrosine phosphorylation of the GHR affects its downregulation is still unknown.

Beta-Transducin Repeats-Containing Proteins

Beta-transducin repeats-containing proteins (β-TrCPs), also termed FWD1, are members of the β-TrCP/Fbw1 subfamily of F-box proteins which constitute the specificity components of the largest known class of E3 ligases (118-122).

E3s are a diverse family of proteins and protein complexes, and they mediate ubiquitination in at least two distinct ways. HECT-type E3s first form an E3-ubiquitin thioester conjugate and then transfer the ubiquitin to the substrate. RING-type E3s, which are characterized by the presence of a RING domain that binds the E2, do not form an E3-ubiquitin conjugate, and they are thought to promote the ubiquitination of the substrate directly by the E2 (123). F box protein is one component of SCF E3 ligase complex which is a four-subunit RING-type E3 and represents the largest family of E3s known to date (124). SCF complexes are composed of the scaffold protein Cul1, the RING-domain protein Roc1 (also termed Rbx1 or Hrt1), the adaptor protein Skp1, and an F box protein that binds the substrate. Roc1 associates with Cul1 and the E2 (125), while Skp1 interacts simultaneously with Cul1 and with the F box protein (126-128). Within SCF complexes, the ligase activity is mediated by the Cullin1 and Roc1 complex, which is tethered via Skp1 to a specificity-conferring F box protein. F box proteins constitute the largest known class of E3 specificity components. F box proteins interact

with Skp1 through the ~40 amino acid F box motif (126, 129) and with substrates through C-terminal protein-protein interaction domains, including WD40 repeats and leucine-rich repeats (124).

β-TrCPs are highly conserved from Xenopus to humans. There are two kinds of β-TrCPs: β-TrCP1, also termed Fbw1a, is predominantly a nuclear protein, whereas, β-TrCP2, also termed HOS or Fbw1b, exhibits mostly cytoplasmic localization (130, 131). Like other F-box proteins, β-TrCPs contain a functionally important F-box motif and carboxy-terminal protein-protein interaction domains, including WD40 repeats, leucine-rich repeats and a cohort of poorly characterized motifs. Substrate specificity of β-TrCPs is determined by protein-protein interaction motifs, through which β-TrCPs recognize and bind to phospho-motifs (DpSGXXpS) in its substrates via WD40 domians (119). In the meantime, β-TrCP proteins recruit the core Skp1-Cul1-Roc1/Rbx1 ubiquitin ligase complex to ubiquitinate the substrates through the F-box motif (124). These interactions ultimately result in degradation of important molecules in various signaling cascades.

The GHR was noted to be ubiquitinated in early purification studies that led to cloning of the receptor cDNA (132). GH-induced GHR ubiquitination has been demonstrated in different cell systems (46, 133). It has been suggested that an intact ubiquitin conjugating system is required for GH-induced endocytosis and degradation of the receptor (44-46, 49, 92, 104, 115), but whether or not GHR ubiquitination itself is

required has been controversial. The putative E3 ubiquitin protein ligases that catalyze GHR ubiquitination have not been identified.

Only two groups have to date reported the involvement of β-TrCPs in ubiquitination and degradation of cytokine receptors both of which harbor the β-TrCP recognition motif, $DSG(X)_{n+2}S$ (50, 134). The cytoplasmic domain of GHR also contains the consensus sequence, DSGRT/AS, which is the β-TrCP recognition motif. This motif is highly conserved in the GHR among species from xenopus to human. What role, if any, of β-TrCPs in GH-induced GHR downregulation is yet to be determined.

Significance and Objective of Dissertation

GH is a potent growth-promoting hormone. It also regulates protein, carbohydrate and fat metabolism. The action of GH is achieved by binding to its cognate receptor GHR, a type Ⅰ transmembrane glycoprotein, followed by the activation of the cytoplasmic assocated non-receptor kinase, JAK2 and phosphorylation of GHR, resulting in the activation of pathways including, but not limited to, MAPK, STAT and PI3K signaling pathways. GH sensitivity is dependent on the abundance of GHR at the cell surface which is regulated by: 1) maturation and trafficking of newly synthesized receptor; 2) proteolytic shredding; 3) constitutive downregulation of the cell surface receptor via endocytosis; 4) GH-induced the cell surface GHR downregulation; and 5) recycling. This dissertation focuses on constitutive downregulation of the cell surface

receptor and GH-induced GHR downregulation. It has been shown that the interaction of N-terminus of JAKs with the membrane proximal region of the OSMR or EpoR, not the kinase activity of JAKs, enhances the surface expression of the receptors. Whereas the increased accumulation of the γ_c subunit at the plasma membrane requires the full-length JAK3, JAK3's its kinase activity is not necessary. Our previous studies demonstrated that JAK2 chaperones precursor GHR and promotes its maturation and helps the surface presentation of GHR by stabilizing it. Here, we first examined the regions of GHR and JAK2 required for the stabilization of GHR on the cell surface by reconstitution of a GHR- and JAK2- deficient human fibrosarcoma cell, γ2A, with either wild-type or mutant GHR and JAK2. We further pursued the effect of GH signaling on GHR fate. Whether JAK2 kinase activity and tyrosine phosphorylation and ubiquitnation of GHR are involved in this process was also investigated in this reconstituted system. These studies will add more evidence to molecular mechanisms of GHR cell surface presentation and GH signaling, and may shed light on GH physiology and pathology, and help better understand mechanisms of cytokine signaling.

DETERMINANTS OF GROWTH HORMONE RECEPTOR DOWNREGULATION

by

LUQIN DENG, KAI HE, XIANGDONG WANG, NING YANG,
CHELLAPPAGOUNDER THANGAVEL, JING JIANG, SERGE Y. FUCHS, AND
STUART J. FRANK

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ABSTRACT

Growth hormone receptor (GHR) is a cytokine receptor family member that responds to GH by activation of the receptor-associated tyrosine kinase, JAK2. We previously showed that JAK2, in addition to being a signal transducer, dramatically increases the half-life of mature GHR, partly by preventing constitutive GHR downregulation. Herein we explored GHR and JAK2 determinants for both constitutive and GH-induced GHR downregulation, exploiting the previously characterized GHR- and JAK2-deficient γ2A reconstitution system. We found that JAK2's ability to protect mature GHR from rapid degradation measured in the presence of the protein synthesis inhibitor, cycloheximide (CHX), depended on the presence of GHR's Box 1 element and the intact JAK2 FERM domain, but not the kinase-like or kinase domains of JAK2. Thus, GHR-JAK2 association, but not JAK2 kinase activity, is required for JAK2 to inhibit constitutive GHR downregulation and enhance GHR half-life. In cells that expressed JAK2, but not cells lacking JAK2, GH markedly enhanced GHR degradation. Like JAK2-induced protection from constitutive downregulation, GH-induced GHR downregulation required the GHR Box 1 element and an intact JAK2 FERM domain. However, a JAK2 mutant lacking the kinase-like and kinase domains did not mediate GH-induced GHR downregulation. Likewise, a kinase-deficient JAK2 was insufficient for this purpose, indicating kinase activity is required. Both lactacystin (a proteasome inhibitor) and chloroquine (a lysosome inhibitor) blocked GH-induced GHR loss. Interestingly, GH-induced GHR ubiquitination, like downregulation, was prevented in cells expressing a kinase-deficient JAK2 protein. Further, a GHR mutant with all cytoplasmic tyrosine residues changed to phenylalanine was resistant to GH-induced GHR ubiquitination and downregulation. Collectively, our data suggest that determinants required for JAK2 to protect mature GHR from constitutive degradation

differ from those that drive GH-induced GHR downregulation. The latter requires GH-induced JAK2 activation and GHR tyrosine phosphorylation, and is correlated to GHR ubiquitination in our reconstitution system.

INTRODUCTION

Growth hormone (GH) is an anterior pituitary-derived peptide hormone that in humans and other vertebrates is important in promotion of growth, regulation of metabolism and energy balance, and likely has a role in longevity (1, 2). GH signaling is initiated by interaction with the cell surface GHR on target tissues and results in activation of several intracellular signaling pathways and expression of a diverse set of genes (3, 4). The GHR is a type 1 glycoprotein that is a member of the cytokine receptor superfamily (5). Like other cytokine receptor family members, GHR couples to a Janus family kinase – specifically, GHR utilizes JAK2 (6). JAK2 is a tyrosine kinase that has no transmembrane domain, but physically and functionally associates with the dimerized transmembrane GHR both in the secretory pathway and at the cell surface by virtue of a proline-rich perimembranous cytoplasmic domain element of the receptor called Box1 and the N-terminal FERM domain of JAK2 (7-14).

An essential determinant of tissue and cellular sensitivity to GH relates to regulation of cell surface GHR abundance and availability, as well as the ability of the GH response to be terminated or dampened. These issues have received much attention in recent years, as mechanisms regulating the biogenesis, trafficking, and downregulation of cell surface receptors in general have become better appreciated. GHR is synthesized in the endoplasmic reticulum, where it rapidly dimerizes as a high-mannose (endoH-sensitive) glycoprotein precursor and then trafficks through the Golgi complex, acquiring

a mature (endoH-resistant) glycosylation pattern and is transported to the cell surface (8, 14-17). Surface GHR abundance in the absence of ligand binding is subject to modulation by two major mechanisms. The receptor is a target for inducible metalloprotease-mediated cleavage in the proximal extracellular domain, which releases the extracellular domain as a GH binding protein, lessens receptor abundance, and reduces sensitivity to subsequent GH stimulation (18-23). The surface GHR can also undergo ligand-independent (constitutive) downregulation via a process that is inhibited by disruption of proteasomal and lysosmal function (24, 25). Recently, we have become interested in the role(s) of JAK2 in regulating GHR surface abundance in the absence of ligand stimulation. We found that JAK2 enhances the level of surface GHR by increasing the efficiency of biogenesis and by enhancing the mature receptor's stability (14, 25). This latter effect is quite significant and presumably relates to effects on the rate of constitutive (ligand-independent) GHR endocytosis and lysosomal downregulation.

GH-induced GHR downregulation proceeds via clathrin coated pit-mediated endocytosis and lysosomal degradation. Intriguingly, elegant studies of Strous and colleagues and others indicate that an intact ubiquitin-proteasome system is required for ligand-induced receptor downregulation and that a cytoplasmic domain sequence downstream of the Box 1 element called the ubiquitin-dependent endocytosis (UbE) motif is required for efficient GHR endocytosis (26-30). Ligand-induced GHR downregulation has been described; however, the degree to which GH enhances constitutive GHR degradation is relatively unclear, with some authors suggesting only a modest change induced by GH (31, 32). Likewise, the role of JAK2 in GH-induced GHR downregulation is also uncertain. Using chemical kinase inhibitors such as the tyrphostin

AG490 and staurosporine, previous studies suggested that blockade of GH-induced JAK2 activation prevents receptor degradation (33, 34); however, these inhibitors lack specificity, making firm conclusions regarding effects of JAK2 per se difficult to draw. In contrast, Strous and collegues found that in Chinese hamster ts20 cell stable transfectants a receptor with a mutation of the Box 1 element underwent GH-induced downregulation similar to a wild-type GHR and concluded that GHR degradation is independent of signal transduction via JAK2 (35).

In this study, we use our well-characterized human fibrosarcoma cell GHR and JAK2 reconstitution system to investigate determinants of constitutive and GH-induced GHR downregulation. We find that constitutive GHR downregulation is strongly inhibited by the association of GHR and JAK2 and that JAK2 kinase activity is not essential for this effect. GH treatment markedly enhances GHR degradation, an effect that relies upon GHR-JAK2 association, but also requires JAK2 kinase activity and GHR tyrosine phosphorylation. We further find that, contrary to previous findings in other systems, GH-dependent ubiquitination of GHR requires activation of JAK2 in that GHR does not become ubiquitinated in cells expressing a kinase-dead JAK2. Likewise, a mutant GHR, in which all tyrosine residues in the intracellular domain are replaced by phenylalanine, though able to bind GH and allow JAK2 activation, does not undergo either GH-induced ubiquitination or degradation. These findings suggest critical roles for both JAK2 activation and GHR tyrosine phosphorylation in GHR downregulation.

MATERIALS AND METHODS

Materials

Recombinant hGH was kindly provided by Eli Lilly Co. (Indianapolis, IN). Cycloheximide, chloroquine, *clasto*-lactacystin β-lactone (referred to as lactacystin), and other routine reagents were purchased from Sigma C**he**mical Co. (St. Louis, MO) unless ot**he**rwise noted. Zeocin was purchased from Invitrogen (Carlsbad, CA). G418 and hygromycin were purchased from Mediatech, Inc. (Cellgro). Fetal bovine serum, gentamicin sulfate, penicillin, and streptomycin were purchased from Biofluids (Rockville, MD).

Antibodies

The rabbit polyclonal anti-phosphorylated JAK2 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal anti-STAT5 and anti-EGFR were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ubiquitin monoclonal antibody was purchased from Stressgen. Anti-phosphorylated STAT5 polyclonal antibody was purchased from Zymed. The 3F10 anti-HA rat monoclonal antibodies was purchased from Roche. Anti-$JAK2_{AL33}$ (directed at residues 746-1129 of murine JAK2) polyclonal serum has been described (66). The rabbit polyclonal antiserum, anti-$GHR_{cyt\text{-}AL47}$, was raised against a bacterially expressed N-terminally His-tagged fusion protein incorporating human GHR residues 271-620 (the entire cytoplasmic domain) and has been previously described (38).

Plasmids

The rabbit GHR cDNA (67) was a gift from Dr. W. Wood, Genentech, Inc., South San Francisco, CA. All JAK2 cDNAs used encode murine JAK2. Ligation of the rabbit GHR cDNA into the pSX expression plasmid has been described previously (55), as have been the generation of pSX-GHR$_{\Delta 278-292}$ (GHR$_{\Delta Box1}$) (9), pEF-BOS-JAK2ΔVIII (JAK2$_{KD}$) (a gift from Dr. P. P. Sayeski, University of Florida) (41, 68), pCI-Neo-JAK2-HA (a gift of Dr. O. Silvennoinen) (69), pMet-IG-pGHR and pMet-IG-MYFc8 (a gift of Dr. J.J. Kopchick, Ohio University) (44), and the pRC/CMV-JAK2$_{\Delta 1-47}$ and pcDNA3.1^{+}-JAK2$_{1-511-HA}$ expression vectors (14).

Cells, cell culture, and transfection

γ2A is a JAK2-deficient human fibrosarcoma cell line kindly provided by Dr. G. Stark (Cleveland Clinic Foundation, Cleveland, OH) (36). A stable γ2A cell line expressing rabbit GHR (γ2A-GHR) and its cell culture conditions have been described (38). A stable γ2A cell line expressing rabbit GHR and mouse JAK2 (γ2A-GHR-JAK2, previously referred to as Clone 14 or C14 cells) was achieved by stable transfection of γ2A-GHR with murine JAK2, as described (14). Stable transfection and maintenance of cell lines γ2A-JAK2, γ2A-GHR$_{\Delta Box1}$ and γ2A-GHR$_{\Delta Box1}$-JAK2 were described previously (23).

Stable γ2A-GHR-JAK2-HA, γ2A-GHR-JAK2$_{1-511-HA}$, γ2A-GHR-JAK2$_{\Delta 1-47}$ and γ2A-GHR-JAK2$_{KD}$ cell lines were achieved by introducing pCI/Neo-JAK2-HA, pcDNA 3.1^{+}-JAK2$_{1-511-HA}$ (which encodes zeocin resistance), pRC/CMV-JAK2$_{\Delta 1-47}$, and pEF-BOS-JAK2ΔVIII plasmids into γ2A-GHR cells individually using Lipofectamine Plus

(Invitrogen) according to the manufacturer's instructions. For transfection of pCI/Neo-JAK2-HA, pRC/CMV-JAK2$_{\Delta 1\text{-}47}$, and pEF-BOS-JAK2ΔVIII, cells were cotransfected with the pcDNA 3.1^{-}-Zeocin plascmid, which encodes zeocin resistance. Cells were selected in DMEM γ2A-GHR growth medium supplemented with 400ug/ml zeocin (after dilution) and screened after 3-5 weeks for full-length or mutant JAK2 expression by blotting with anti-JAK2$_{AL\text{-}33}$ or 3F10 antibody. γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 were generated by stable transfection of γ2A-JAK2 cells with pMet-IG-pGHR or pMet-IG-MYFc8 respectively in the same fashion as the generation of γ2A-GHR$_{\Delta Box1}$-JAK2 (23). These stable cell lines were maintained in the same media as used for γ2A-GHR-JAK2.

Cell stimulation, protein extraction, immunoprecipitation, electrophoresis, and immunoblotting

Serum starvation of cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V, Roche Molecular Biochemicals, Indianapolis, IN) for serum in their respective culture media for 16-20 hr prior to experiments. Adherent cells were stimulated with human GH (500 ng/ml) in Dulbecco's modified Eagle's medium (low glucose) with 0.5% (w/v) bovine serum albumin at 37°C. Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline (PBS) in the presence of 0.4 mM sodium orthovanadate (PBS-vanadate). Cells were harvested by scraping in ice-cold PBS-vanadate and pelleted cells were collected by brief centrifugation. For protein extraction, pelleted cells were solubilized for 30 min at 4°C in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0),

100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 10 μg/ml aprotinin), as indicated. For experiments in which ubiquitination was assayed, N-ethylmaleimide (5 mM) was added to the lysis and immunoprecipitation buffers to prevent post-lysis deubiquitination of proteins. After centrifugation at 15,000 × g for 15 min at 4°C, the detergent extracts were electrophoresed under reducing conditions or subjected to immunoprecipitations, as indicated. For immunoprecipitation with anti-$GHR_{cyt\text{-}AL47}$, 3 μl of antiserum was used per precipitation. Protein-A Sepharose (Amersham Biosciences) was used to adsorb immune complexes and, after extensive washing with lysis buffer, SDS sample buffer eluates were resolved by SDS-PAGE and immunoblotted as indicated. Resolution of proteins by SDS-PAGE, Western transfer of proteins, and blocking of Hybond-ECL (Amersham Biosciences) with 2% bovine serum albumin were performed as previously described (14, 37, 38, 70). Immunoblotting with anti-$GHR_{cyt\text{-}AL47}$ (1:4000), anti-$JAK2_{AL33}$ (1:4000), anti-EGFR (1:1000), anti-STAT5 (1:1000), 3F10 (100 ng/ml), anti-ubiquitin (1:1000), anti-P-JAK2 (1:1000), or anti-P-STAT5 (1:1000) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:10000), anti-rat secondary antibodies (1:2000), or anti-mouse secondary antibodies (1:5000) and ECL detection reagents (SuperSignal West Pico chemiluminescent substrate; all from Pierce Chemical Co., Rockford, IL) and stripping and reprobing of blots were accomplished according to the manufacturer's suggestions.

D*egradation of GHR after blockade of protein synthesis ("cycloheximide chase")*

Cells were grown to 90% confluence in 6-well plates, serum-starved overnight, and then incubated with cycloheximide (CHX) (20 g/ml) for 0-5 hr. The treatments

were ended by washing with ice-cold PBS and cells were harvested and detergent extracts prepared as described above. Cell lysates were resolved by SDS–PAGE and blotted with anti-$GHR_{cyt\text{-}AL47}$, anti-$JAK2_{AL33}$, or anti-EGFR.

Effects of proteasome and lysosome inhibitors on GH-induced GHR degradation

Cells were grown to 90% confluence in six-well plates and serum starved overnight. After incubation with lactacystin (15 M) or chloroquine (100 M) for 2 h, GH (500 ng/ml) or vehicle was added for 3 h. Cell lysates were resolved by SDS-PAGE and blotted with anti-$GHR_{cyt\text{-}AL47}$.

Densitometric and statistical analysis

Immunoblots were scanned using a high-resolution scanner (Hewlett-Packard Co.). Densitometric quantification of images exposed in the linear range was performed using an image analysis program, Image J (developed by W. S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data of densitometry from several experiments are displayed as mean ± SE.

RESULTS

JAK2 association, but not the JAK2 kinase domain, is required for stabilization of mature GHR.

γ2A is a human fibrosarcoma cell that lacks both GHR and JAK2 (36, 37). We previously demonstrated that stable reconstitution with GHR and JAK2 (γ2A-GHR-JAK2 cells), when compared to GHR alone (γ2A-GHR cells), allowed GH-induced signaling and enhanced surface GHR abundance (14, 25). This enhanced abundance was

substantially accounted for by the ability of JAK2 to selectively stabilize the mature form of the receptor. We sought to determine regions of GHR and JAK2 required for this stabilizing effect of JAK2.

To assess GHR degradation rates, we used cycloheximide (CHX) to inhibit new protein synthesis and followed the fate of previously synthesized GHR by immunoblotting with an anti-GHR serum that recognizes the receptor cytoplasmic domain (38). In the experiment shown in Figure 1, four stable transfectant cell lines were compared. These included the previously described γ2A-GHR and γ2A-GHR-JAK2 cells as well as newly prepared stable clones that express a GHR with an in-frame internal deletion of the Box 1 element (diagrammed in Figure 1A), either in the absence (γ2A-$GHR_{\Delta Box1}$) or presence (γ2A-$GHR_{\Delta Box1}$-JAK2) of JAK2. We previously reported that this GHR mutant lacking Box 1 is structurally intact, but cannot interact with JAK2 or signal in response to GH (9). Serum-starved cells were treated with CHX for 0-5 h, as indicated (Figure 1B), and detergent-extracted proteins were resolved by SDS-PAGE and anti-GHR immunoblotted. With increasing CHX treatment duration, precursor (P) abundance decreased rapidly to a similar degree in all four cell lines. This result indicates that the precursor is quite unstable, consistent with previous data (25), and that precursor degradation rate was unaffected by the presence of JAK2 or its ability to associate with GHR. These data are shown graphically in Figure 1C, left panel, in which the results of multiple such experiments were evaluated densitometrically and the receptor remaining after CHX treatment is expressed as a percentage of the level in untreated cells.

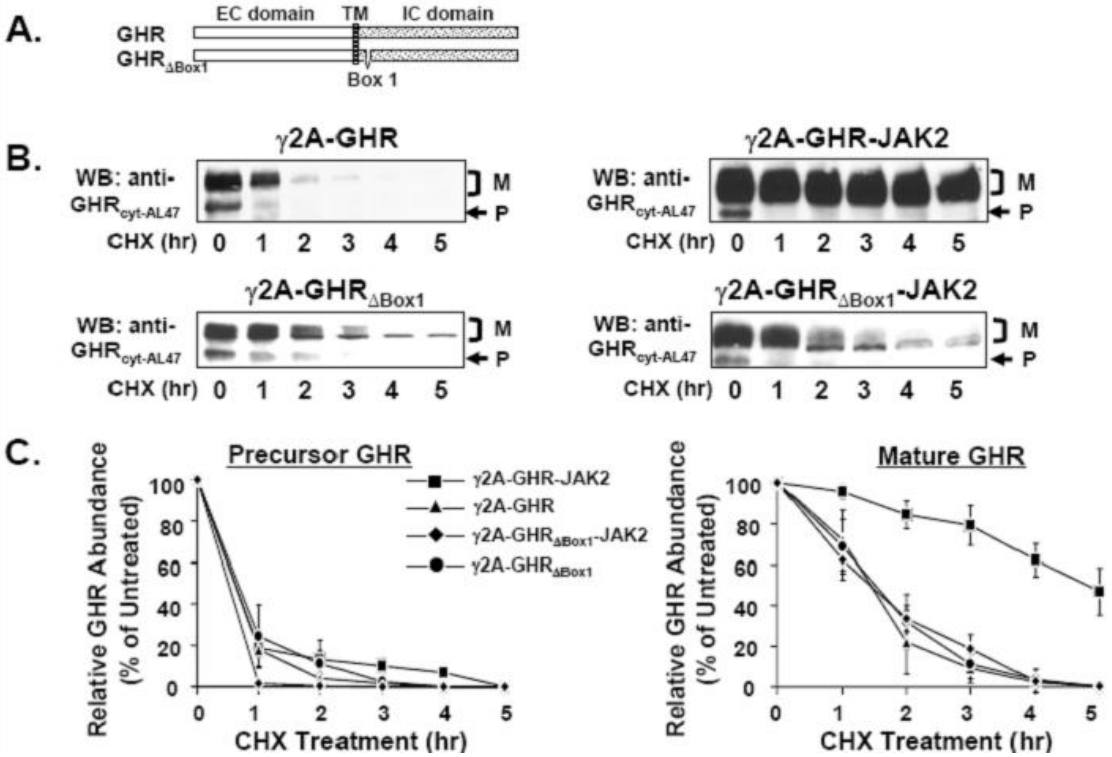

Figure 1. The Box 1 element of GHR is required for JAK2's stabilizing effect on mature GHR.

A, Diagram of wild-type GHR compared to GHR $_{Box1}$. EC, extracellular; TM, transmembrane; IC, intracellular. B, CHX-induced GHR loss. Serum-starved γ2A-GHR, γ2A-GHR-JAK2, γ2A-GHR$_{\Delta Box1}$ and γ2A-GHR$_{\Delta Box1}$-JAK2 cells were treated with CHX (20 μg/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR$_{AL47}$. The positions of the mature (*bracket*) and precursor (*arrowhead*) GHR forms are indicated. The data shown are representative of three such experiments. C, Densitometric quantitation of anti-GHR blots, including those in B. The mature GHR abundance at time 0 was considered 100%, respectively, for each experiment (mean ± SE; n = 3 independent experiments).

In contrast to the precursor, the kinetics of mature (M) GHR loss after CHX treatment differed substantially among the cells (Figure 1B and 1C, right panel). As we previously demonstrated (25), the stability of wild-type mature GH receptor was greatly enhanced by JAK2 (compare γ2A-GHR ($t_{½}$ ~ 1.5 h) vs. γ2A-GHR-JAK2 ($t_{½}$ > 4 h)). However, in cells that expressed the Box1-deleted GHR, the stability of the receptor was similar to that in γ2A-GHR and was unaffected by expression of JAK2 (compare γ2A-$GHR_{\Delta Box1}$ and γ2A- $GHR_{\Delta Box1}$-JAK2 with the other cells and with each other). Thus, the GHR Box1 element is required for JAK2's stabilizing effect on mature GHR.

We sought to confirm this model in a reciprocal fashion by testing the capacity of JAK2 mutants to stabilize the mature GHR (Figure 2). In addition to the GHR Box1 element, GHR-JAK2 association requires an intact FERM domain, which resides in the N-terminal 450 residues of JAK2 (14, 39, 40). Removal of the N-terminal 47 residues of JAK2 ($JAK2_{\Delta 1-47}$), which includes the first FERM subdomain, renders JAK2 unable to physically or functionally interact with GHR (14). Another previously characterized JAK2 mutant, $JAK2_{1-511-HA}$, includes the first 511 residues and thus has the FERM domain, but lacks the pseudokinase and kinase domains (14) (see diagram in Figure 2A). CHX treatment of cells stably expressing wild-type GHR and $JAK2_{\Delta 1-47}$ (γ2A-GHR-$JAK2_{\Delta 1-47}$ cells) yielded rapid loss of mature GHR (Figure 2B, left panel and graph in Figure 2C), indicating that this FERM domain mutant JAK2 was unable to stabilize the mature GHR. In contrast, the GHR in γ2A-GHR-$JAK2_{1-511-HA}$ cells was substantially stabilized in the same assay (Figure 2B, right panel and Figure 2C). Collectively, these data indicate that association of JAK2 with GH receptor, mediated by the receptor Box 1 element and the JAK2 FERM domain, is required for enhanced stability of the mature

GH receptor. Conversely, the pseudokinase and kinase domains of JAK2 are not required for this effect.

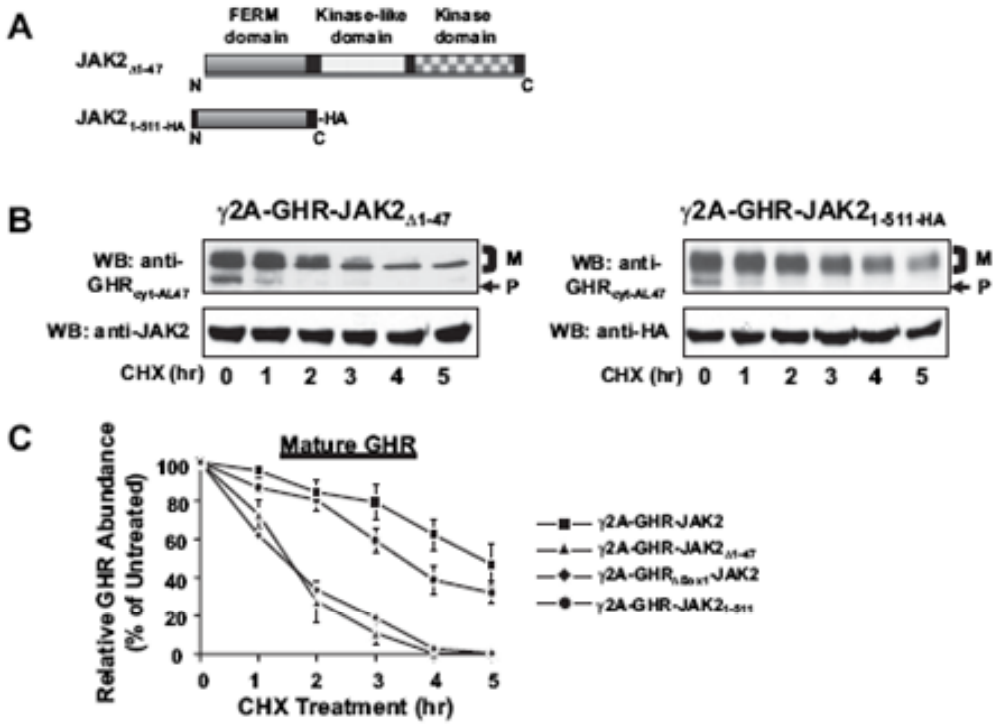

Figure 2, An intact N-terminus, but not the pseudokinase and kinase domains, of JAK2 is required to stabilize mature GHR.

A, Diagram of $JAK2_{\Delta 1\text{-}47}$ compared to $JAK2_{1\text{-}511\text{-}HA}$. B, Serum-starved γ2A-GHR-$JAK2_{\Delta 1\text{-}47}$, γ2A-GHR-$JAK2_{1\text{-}511\text{-}HA}$, and γ2A-GHR-JAK2 cells were treated with CHX (20 μg/ml) for 0–5 h, as described in Fig 1. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47} and anti-$JAK2_{AL33}$. C, Densitometric quantitation of anti-GHR blots was done as above. (mean ± SE; n = 3 independent experiments).

GH markedly enhances GHR downregulation in a JAK2-dependent fashion.

The experiments in Figures 1-2 addressed the effect of JAK2 on constitutive GHR downregulation. There are conflicting reports concerning the degree to which GH affects the rate of GHR degradation and whether GHR-JAK2 association influences this. We used our stably transfected cells to address these issues. First, serum-starved γ2A-GHR-JAK2 cells were treated with CHX alone vs. CHX plus GH for 0-5 h. Anti-GHR blotting of cell extracts revealed marked GH-induced enhancement of GHR loss. Densitometric evaluation of three independent experiments like that shown in Figure 3 revealed that GH treatment in this context reduced the GHR half-life from > 4 h to roughly 1 h (Figure 3).

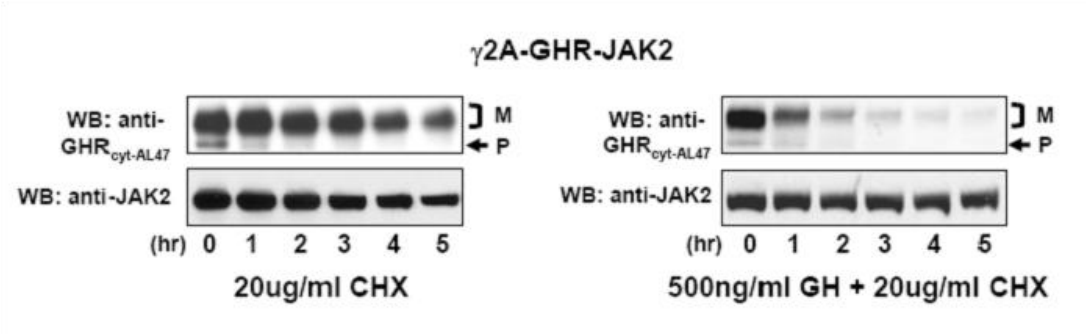

Figure 3, GH markedly accelerates degradation of mature GHR.
Serum-starved γ2A-GHR-JAK2 cells were treated with CHX (20 μg/ml) alone or co-treated with CHX (20 μg/ml) plus GH (500 ng/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47} and anti-$JAK2_{AL33}$. The data shown are representative of three such experiments.

To examine the requirement for JAK2 for GH-dependent receptor loss, JAK2-deficient (γ2A-GHR) and JAK2-replete (γ2A-GHR-JAK2) cells were compared (Figure

4A). In cells that lacked JAK2, GH caused no loss of mature or precursor GHR (Figure 4A, left panel). In contrast, GH treatment caused marked loss of mature GH receptor in γ2A-GHR-JAK2 cells (Figure 4A, right panel), suggesting that JAK2 expression is required for this effect of GH. (In other experiments (not shown), GH-induced GHR degradation in this setting was dose-dependent.) Blotting for STAT5 and EGFR in the same extracts served as a loading control. Notably, GH also caused loss of mature GHR in CHO cells stably expressing GHR (Figure 4B, left panel) and in 3T3-F442A cells, murine preadipocytes that endogenously express GHR (Figure 4B, right panel).

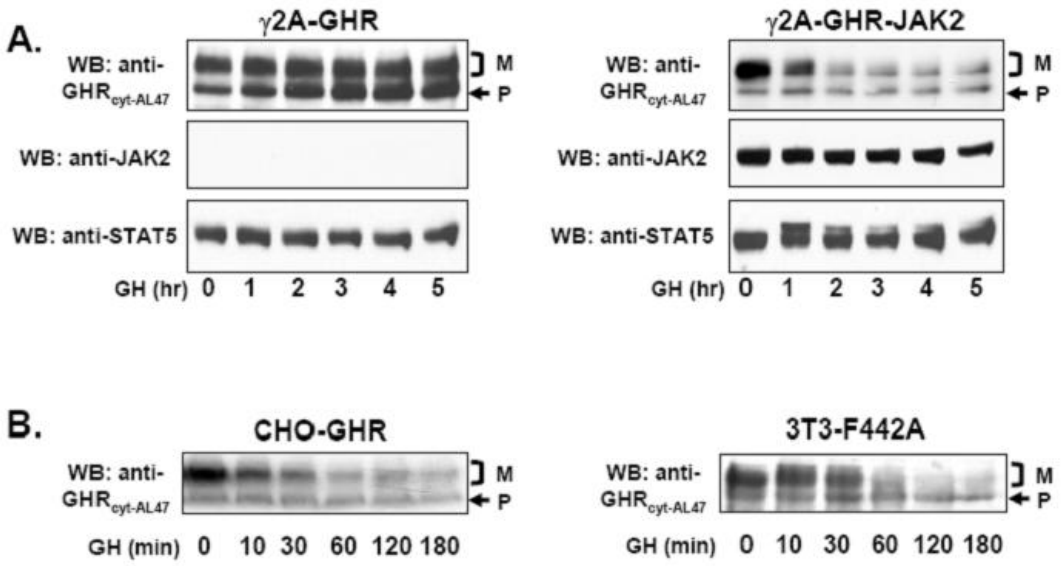

Figure 4, JAK2 is required for GH to enhance GHR downregulation.

A, Serum-starved γ2A-GHR and γ2A-GHR-JAK2 cells were treated with GH (500 ng/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47}, anti-$JAK2_{AL33}$, and anti-STAT5. The data shown are representative of three such experiments. B, Serum-starved CHO-GHR and 3T3-F442A cells were treated GH (500 ng/ml) for 0–3 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47}. The data shown are representative of three such experiments.

GH-dependent GHR downregulation requires GHR-JAK2 association and JAK2 kinase activity.

The data in Figure 4A indicate that JAK2 expression is required for GH-induced GHR downregulation, but do not indicate what role JAK2 plays in this process. To approach this question, we first assessed whether GHR-JAK2 association was required by comparing γ2A-GHR-JAK2, γ2A-GHR$_{\Delta Box1}$-JAK2, and γ2A-GHR-JAK2$_{\Delta 1-47}$ cells (the first having wild-type GHR and JAK2, the second having the Box1-deleted GHR and wild-type JAK2, and the third having wild-type GHR and the FERM domain-disrupted JAK2). Serum-starved cells treated with GH were evaluated by anti-GHR blotting (Figure 5A). In contrast to the marked GH-dependent mature GHR downregulation seen in γ2A-GHR-JAK2, GH did not cause mature or precursor GHR loss in cells bearing either the Box1- or FERM-deleted GHR or JAK2, respectively (Figure 5B). These data strongly suggest that association with JAK2 is required for this GH-dependent downregulation of GHR.

We next evaluated whether GHR-JAK2 association is sufficient to allow GH-induced receptor degradation. Cells coexpressing wild-type GHR and either wild-type (HA-tagged) JAK2 (γ2A-GHR-JAK2-HA) or the JAK2 mutant encoding only the first 511 residues (γ2A-GHR-JAK2$_{1-511-HA}$) were compared (Figure 6). As expected, expression of JAK2-HA allowed marked GH-dependent receptor loss. However, in cells expressing JAK2$_{1-511-HA}$, GH did not cause GHR disappearance. Thus, while required, the FERM domain of JAK2 is not sufficient to allow GH-dependent receptor downregulation in this system.

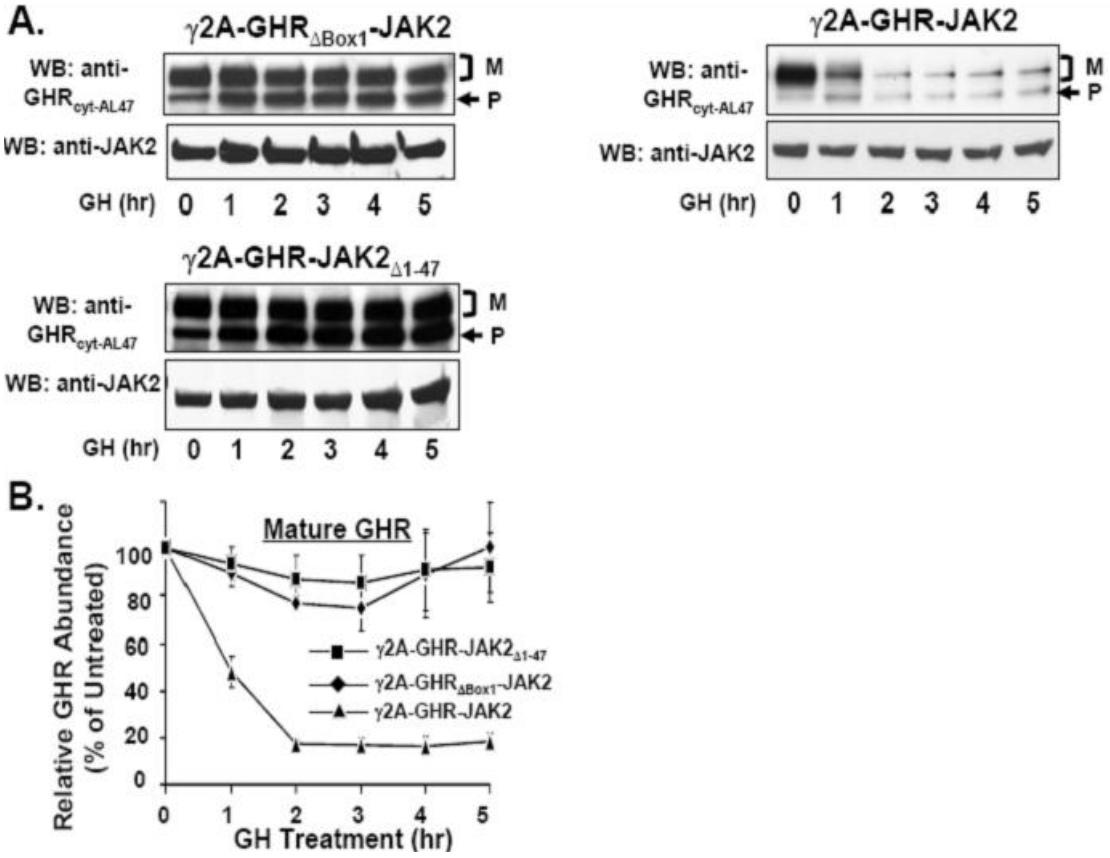

Figure 5, GH-induced GHR loss requires GHR-JAK2 association.

A, Serum-starved γ2A-GHR$_{\Delta Box1}$-JAK2, γ2A-GHR-JAK2$_{\Delta 1-47}$ and γ2A-GHR-JAK2 cells were treated with GH (500 ng/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR$_{AL47}$ and anti-JAK2$_{AL33}$. B, Densitometric quantitation of the anti-GHR blots was done as in Figure 1 (mean ± SE; n = 3 independent experiments).

We sought to investigate the role of JAK2 catalytic activity in our genetically tractable system (Figure 7). $JAK2_{KD}$ has point mutations at residues 1020 (W to G) and 1024 (E to A) that abrogate its phosphotransferase activity (41) (Figure 7A). GH treatment of γ2A-GHR-$JAK2_{KD}$ failed to downregulate GHR (Figures 7B,C), indicating that JAK2 protein kinase activity is required for this effect.

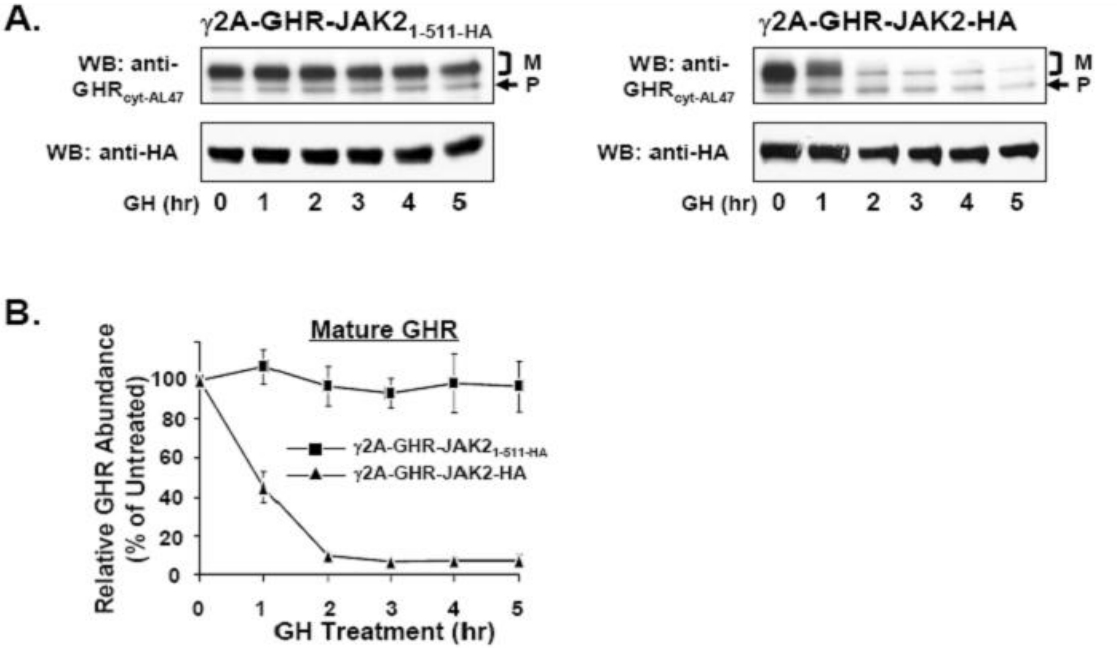

Figure 6, GHR-JAK2 association is not sufficient for GH-induced GHR degradation.

A, Serum-starved γ2A-GHR-$JAK2_{1-511-HA}$ and γ2A-GHR-JAK2-HA cells were treated with GH (500 ng/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-HA antibody, 3F10. B, Densitometric quantitation of the anti-GHR blots was measured as described before (mean ± SE; n = 3 independent experiments).

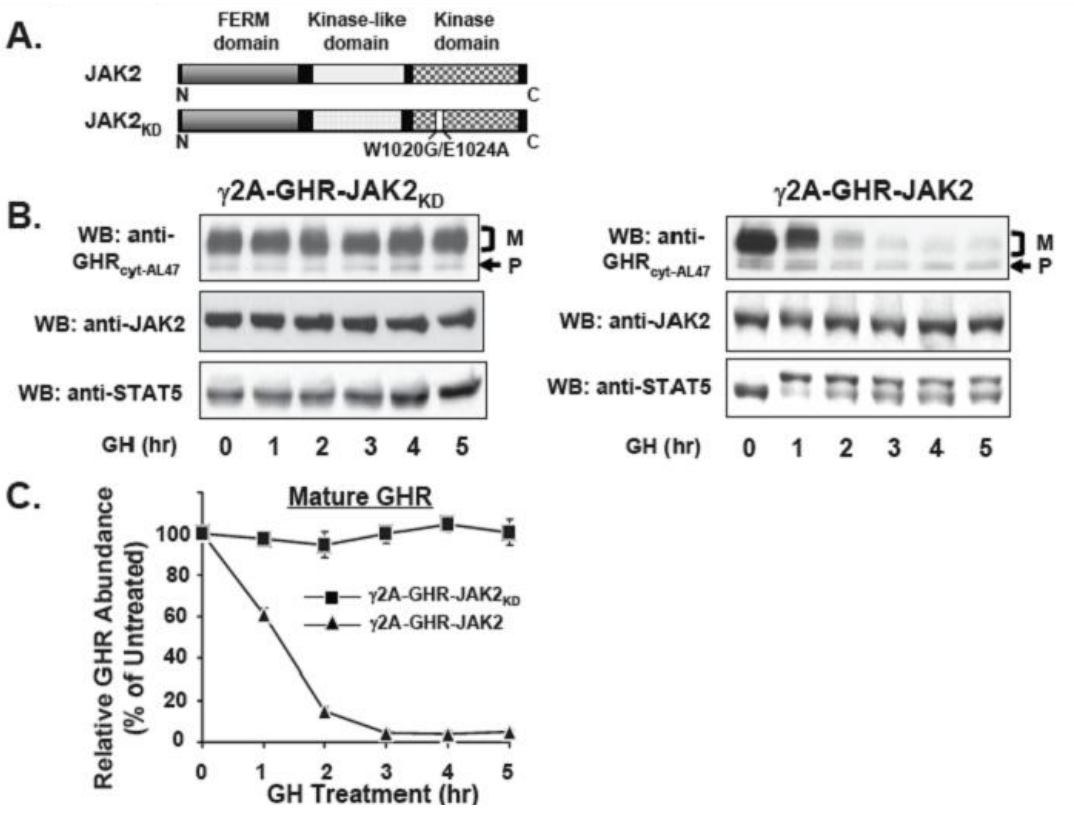

Figure 7, JAK2 catalytic activity is required for GH-induced GHR downregulation.

A, Diagram of $JAK2_{KD}$ compared to JAK2. B, Serum-starved γ2A-GHR-JAK2 and γ2A-GHR-$JAK2_{KD}$ cells were treated with GH (500 ng/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47}, anti-$JAK2_{AL33}$, and anti-STAT5. C, Densitometric quantitation of the anti-GHR blots was done as above (mean ± SE; n = 3 independent experiments).

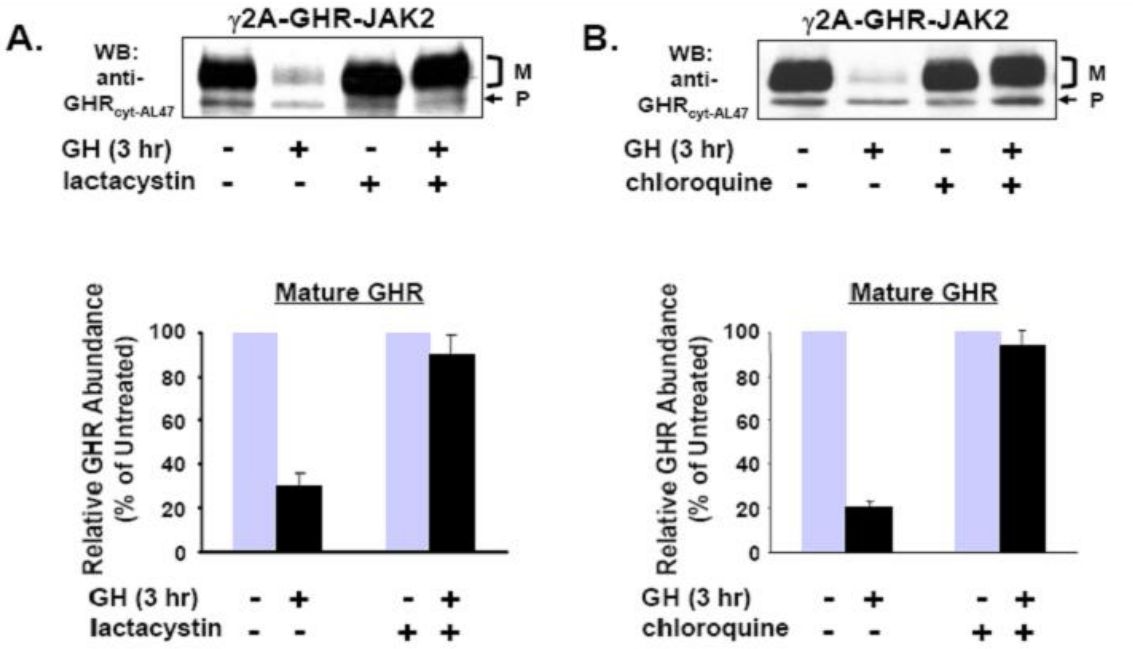

Figure 8, Effects of proteasome and lysosome inhibitors on GH-induced GHR downregulation.

A,B, Serum-starved γ2A-GHR-JAK2 cells were pretreated with either lactacystin (A) or chloroquine (B) or their vehicles prior to stimulation with GH for 3 h. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47}. Densitometric quantitation of the anti-GHR blots, including those in the upper panels, is shown in the lower panels of A and B. The mature GHR abundance at time 0 was considered 100% (mean ± SE; n = 2 independent experiments in A and 3 independent experiments in B).

Effects of proteasome and lysosome inhibitors on GH-induced GHR downregulation.

Using the γ2A reconstitution system, we previously showed that proteasome inhibitors and lysosome inhibitors both blocked constitutive (CHX-induced) GHR downregulation (25). In the experiments shown in Figure 8, we tested the effects of the proteasome inhibitor, lactacystin, and the lysosome inhibitor, chloroquine, on GH-induced GHR degradation in this same system. Serum-starved γ2A-GHR-JAK2 cells were pretreated with lactacystin (A) or chloroquine (B) or their vehicles and then exposed to GH or vehicle for 3 h. Anti-GHR blotting of detergent extracts revealed that the profound GH-induced GHR loss was blocked by pretreatment with each inhibitor. Densitometric quantitation of several such experiments confirmed these results. As these data were obtained with chemical inhibitors, we interpret them cautiously. However, they are consistent with the notion that, like constitutive GHR downregulation, inhibition of either proteasome activity or lysosome activity blocks efficient GH-induced GHR downregulation.

Tyrosine phosphorylation of GHR is required for its ubiquitination and downregulation by GH.

GH-induced GHR ubiquitination has previously been demonstrated in other cell systems (31, 42). The role of JAK2 signaling in allowing this receptor modification has yet to be directly tested, however. We used our reconstitution system to probe this issue (Figure 9A). For these experiments, GHR was immunoprecipitated, resolved by SDS-PAGE, and blotted with an anti-ubiquitin antibody. Stimulation of γ2A-GHR-JAK2 cells with GH indeed yielded a diffuse, slower migrating species detected by anti-ubiquitin

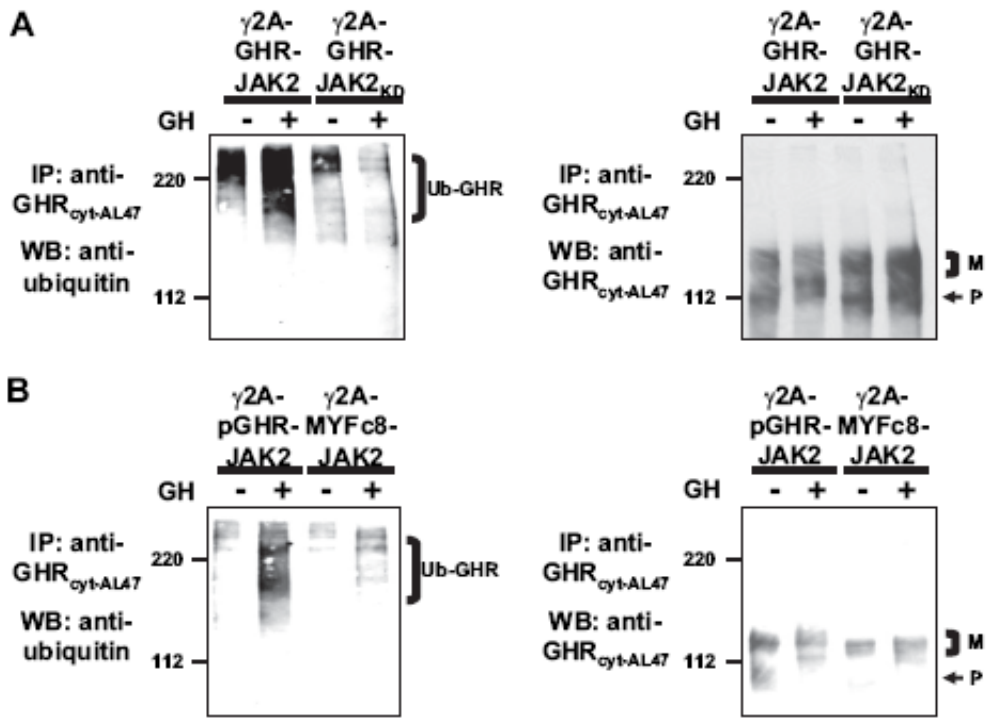

Figure 9, GH-induced GHR ubiquitination requires JAK2 kinase activity and GHR tyrosine phosphorylation.

A,B, Serum-starved γ2A-GHR-JAK2 vs. γ2A-GHR-JAK2$_{KD}$ cells (A) and γ2A-pGHR-JAK2 vs. γ2A-MYFc8-JAK2 cells (B) were treated with GH or vehicle for 30 min. Detergent extracts were immunoprecipitated with anti-GHR$_{AL47}$. Eluates were resolved by SDS-PAGE and sequentially immunoblotted with anti-ubiquitin antibody (left panels) and anti-GHR$_{AL47}$ (right panels). The positions of the ubiquitinated GHR (left panels) and mature (*bracket*) and precursor (*arrowhead*) GHR forms (right panels) are indicated. The data shown are representative of 2 such experiments.

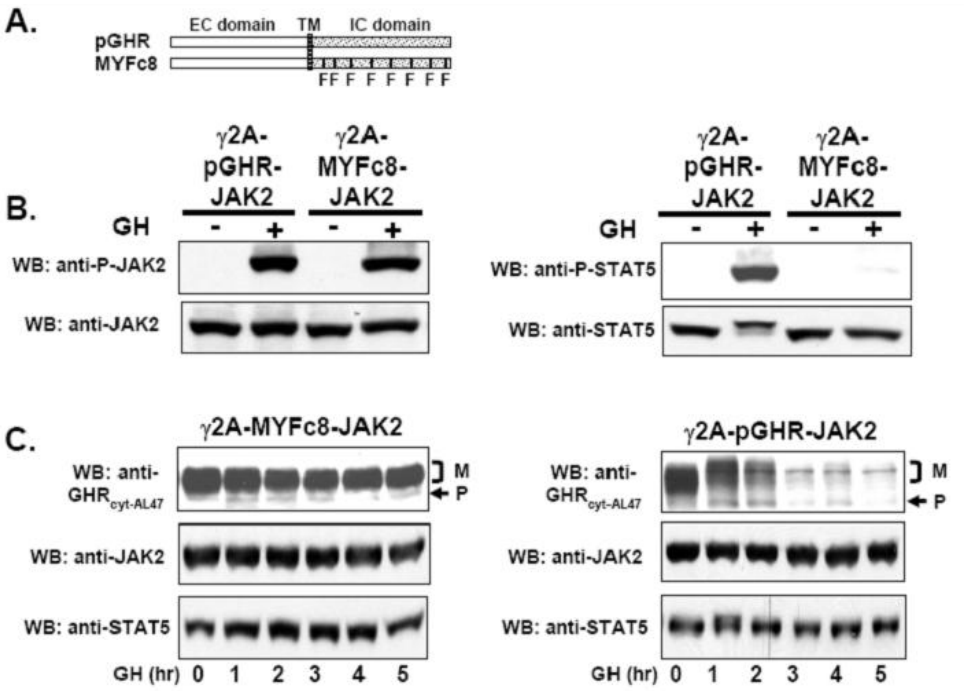

Figure 10, Tyrosine phosphorylation of GHR is required for its GH-induced degradation.

A, Diagram of wild-type porcine GHR compared to γ2A-MYFc8-JAK2. B, GH induces tyrosine phosphorylation of JAK2, but not STAT5, in γ2A-MYFc8-JAK2 cells. Serum-starved γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells were treated with GH or vehicle for 15 minutes. Detergent extracts were resolved by SDS-PAGE and sequentially blotted with anti-pJAK2 and JAK2 (left panel) or anti-pSTAT5 and STAT5 (right panel). The data shown are representative of three such experiments. C, Serum-starved γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells were treated with GH (500 ng/ml) for 0–5 h, as indicated. Detergent extracts were resolved by SDS-PAGE and blotted with anti-GHR_{AL47}, anti-$JAK2_{AL33}$, and anti-STAT5. The data shown are representative of three such experiments.

antibody (Figure 9A, left panel). This is consistent with the migration of the ubiquitinated GHR reported previously (26, 42). In the same experiment, we asked whether JAK2 kinase activity was required for GH-induced receptor ubiquitination. In contrast to the cells that harbor wild-type JAK2, GH did not increase GHR ubiquitination in γ2A-GHR-JAK2$_{KD}$ cells, in which GHR was not downregulated by GH (Figure 9A, left panel). Stripping and reprobing revealed similar GHR abundance in the two cells (Figure 9A, right panel). Along with the data in Figure 7, these data strongly suggest that GH-induced JAK2 kinase activity is required for GH-induced receptor ubiquitination and downregulation. These results differ substantially from those reported by Alves dos Santos, et al (35), from which it was concluded that GH-induced GHR ubiquitination (and downregulation) occurred even if GH-induced JAK2 signaling was blocked; this point is further addressed in the Discussion.

The data in Figures 5-7 indicate that GHR-JAK2 association and intact JAK2 kinase activity are required for GH-induced GHR downregulation. There are multiple cellular proteins that become tyrosine phosphorylated in response to GH in a JAK2-dependent fashion; these include the GHR, JAK2 itself, STAT5, and others (3, 4). Elegant studies using a GHR in which all cytoplasmic domain tyrosine residues were mutated to phenylalanine revealed that GHR tyrosine phosphorylation is not required for GH-induced JAK2 activation, but is necessary for STAT5 tyrosine phosphorylation and action (43, 44). We tested whether this mutant GHR could undergo GH-induced downregulation.

For these experiments, we used the MYFc8 porcine GHR in which all eight cytoplasmic tyrosine residues were changed to phenylalanine (44) (a gift of Drs. E. List and J.J. Kopchick) (Figure 10A). For control, we used the wild-type porcine GHR

(pGHR). Each was stably expressed in γ2A-JAK2 cells to yield γ2A-MYFc8-JAK2 and γ2A-pGHR-JAK2 cells. Consistent with the previous reports in other cell systems, GH stimulation acutely activated JAK2 and caused its tyrosine phosphorylation, but did not cause tyrosine phosphorylation of STAT5 (Figure 10B). As expected, GH treatment caused loss of the wild-type porcine GHR (Figure 10C, right panel), just as occurred in cells expressing rabbit and mouse GHR (Figures 3-8). Notably, however, GH did not substantially downregulate the mutant GHR in γ2A-MYFc8-JAK2 cells. This result strongly suggests that either receptor tyrosine phosphorylation itself or the association of an important trafficking molecule with the tyrosine phosphorylated GHR enables the receptor to undergo degradation in response to GH.

Results in Figure 9A demonstrated that GH-induced GHR ubiquitination required the ability of JAK2 to be active as a kinase. We further asked whether GHR tyrosine phosphorylation is required for receptor ubiquitination (Figure 9B). GH caused ubiquitination of wild-type porcine GHR, but not the MYFc8 mutant receptor. Thus, both GH-induced receptor ubiquitination and downregulation required not only activation of JAK2, but also tyrosine phosphorylation of the receptor itself.

DISCUSSION

In addition to the levels of JAK2, STAT5, and other intracellular GH signaling elements, cell surface GHR abundance is an important factor that modulates biological responses to GH at target tissues (3, 4, 20, 22, 45-48). JAK2 is the critical proximal signal transducer for GH action; in cells that lack JAK2 or in which JAK2 cannot associate with the GHR, GH is unable to trigger the major signaling pathways downstream of the receptor (6, 9-13). We recently uncovered additional effects of JAK2

on GHR. By virtue of its interaction with GHR, JAK2 impacts GHR biogenesis, the stability of the mature cell surface receptor, and susceptibility of the receptor to metalloproteolysis ((14, 23, 25) and Loesch et al, submitted).

In the current study, we further explored the effects of JAK2 on stability of the mature GHR and examined GHR and JAK2 determinants for GH-induced receptor downregulation. We found that, like its effects on biogenesis and metalloprotease sensitivity, the capacity of JAK2 to prevent constitutive GHR downregulation requires the ability of the two molecules to associate. This conclusion is based on the fact that such association is disrupted by mutation in either JAK2 ($JAK2_{\Delta 1\text{-}47}$) or GHR ($GHR_{\Delta Box1}$). Further, the protective effect of JAK2 does not require either the pseudokinase or kinase domains (the $JAK2_{1\text{-}511}$ mutant). Thus, the ability of JAK2 to bind the receptor is apparently sufficient to fulfill this role.

We also studied whether JAK2 affected GH-induced GHR downregulation. We first established that GH in our reconstitution system greatly accelerated the rate of GHR degradation. Likewise, we observed a robust GH-induced GHR loss in a separate reconstitution system (CHO-GHR in Figure 4B) and in cells that endogenously express GHR and JAK2 (3T3-F442A in Figure 4B and H4IIE hepatoma cells in ref (49)).

GH-induced GHR downregulation in our reconstitution system was quite dependent on JAK2; GH promoted no loss of GHR in cells that lack JAK2. In contrast, GH-induced GHR degradation was substantial in cells reconstituted with JAK2 (Figure 4A). This is an important result, as it addresses an issue about which there is some disagreement in the literature and which has not previously been addressed in the definitive fashion allowed by use of the JAK2 reconstitution system employed in our studies. Alves dos Santos, et al (35) found that cells that express a GHR in which all four

Box1 proline residues are mutated displayed no difference in GH-induced GHR degradation in pulse chase experiments, although the GH effect was rather modest even for wild-type receptor in the cell system used in those studies (32). To the contrary, our data in Figure 5A with a previously characterized (9, 23) Box1 deletion mutant GHR indicated that this receptor was not downregulated at all in response to GH, despite the presence of JAK2. Thus, we conclude that GH-induced GHR degradation depends on the presence of JAK2 and its ability to associate with GHR.

Our mutational studies of JAK2 confirmed that JAK2 association was required (γ2A-GHR-JAK2$_{\Delta 1\text{-}47}$; Figure 5A), but was not sufficient (γ2A-GHR-JAK2$_{1\text{-}511\text{-}HA}$; Figure 6) for GH-induced GHR downregulation. Interestingly, this is distinctly different from our findings about constitutive receptor downregulation, for which GHR-JAK2 association was required and sufficient to protect the receptor (above and Figure 2). Further, GH-induced GHR downregulation was not allowed by a JAK2 mutant in which only the kinase domain was targeted (γ2A-GHR-JAK2$_{KD}$; Figure 7). This result strongly implicates GH-induced JAK2 kinase activity as necessary for GH-induced GHR downregulation.

Strous and colleagues first demonstrated GH-induced GHR ubiquitination and proposed that an intact ubiquitin conjugating system was required for GH-induced endocytosis and degradation of the receptor (29), but later concluded that receptor ubiquitination per se was not required for GH-induced GHR internalization (30). This conclusion was drawn from experiments performed with a truncated (rather than full-length) GHR in which all remaining lysine residues were replaced with arginine (and thus were not available for ubiquitin conjugation) and which was still internalized in response to GH (30). Notably, GH-induced GHR degradation was not monitored in that

study; thus, conclusions concerning whether GHR ubiquitination was required for downregulation to occur could not be drawn (30). Further work in the same system (referred to above) indicated that GH-induced GHR ubiquitination proceeded even for a GHR with a mutated Box1 element, suggesting to the authors that it was independent of GH-induced JAK2 signaling (35).

In the current study, we also detected GH-induced GHR ubiquitination in cells that express wild-type GHR and JAK2 (Figure 9). However, our finding that this modification was not observed in cells that harbored a kinase-deficient JAK2 is in sharp contrast to those mentioned above. Furthermore, our data indicate that the lack of GH-induced GHR ubiquitination in γ2A-GHR-JAK2$_{KD}$ cells correlates to the inability of GH to cause receptor downregulation in those cells. This relationship is correlative, but not necessarily causal, however, and further experiments would be needed to establish that ubiquitination is required for downregulation of the receptor. We are uncertain as to why our conclusions that GH-dependent JAK2 kinase activation is necessary for GH-induced GHR ubiquitination and downregulation differ from those drawn previously from studies of GH-induced ubiquitination and degradation of a Box 1-mutated GHR in the temperature-sensitive Chinese hamster fibroblast cellular system reported by Alves dos Santos, et al (35).

Our experiments with a GHR with all cytoplasmic tyrosine residues mutated to phenylalanine further tested the basis for the requirement for intact JAK2 kinase function for GH-induced GHR ubiquitination and degradation. Interestingly, we found that GH-induced JAK2 kinase activation, although required, was insufficient for these effects on GHR. Apparently, GHR itself needs to undergo tyrosine phosphorylation in order to become ubiquitinated (Figure 9B) and downregulated (Figure 10). Although other

explanations are possible, these data suggest to us that tyrosine phosphorylation of the receptor cytoplasmic domain allows direct binding via a phosphorylated tyrosine(s) to a protein or proteins involved in these processes or that the tyrosine phosphorylated receptor is a better target for another modification (eg., phosphorylation at serine or threonine residues) by other enzymes that then enhance the receptor's susceptibility to ubiquitination and degradation.

Tyrosine phosphorylation is critical to downregulation of other surface receptors. Epidermal growth factor receptor (EGFR), for example, undergoes EGF-induced autophosphorylation, which allows association with ubiquitin ligase activity and EGFR ubiquitination; this allows interaction with proteins in the endocytic/lysosomal pathway and ultimate degradation in lysosomes (50). Indeed, the tyrosine phosphorylated GHR binds well to several SH2-containing proteins involved in signaling and trafficking (17, 51-54). We previously found that the SH2-containing protein tyrosine phosphatase, SHP-2, binds specifically to a tyrosine phosphorylated GST fusion protein incorporating the distal 40% of the human GHR cytoplasmic domain, which harbors four tyrosine residues (55). Stofega, et al demonstrated that mutation of one of these tyrosine residues in the rat GHR (corresponding to Y-577 in the human and rabbit GHR) markedly reduced SHP-2 association and prolonged GH-induced signaling (52). This mutant GHR was also downregulated somewhat less rapidly than the wild-type GHR, but the effect was subtle compared with the dramatic lack of GH-induced degradation of the MYFc8 receptor we observed in Figure 10C. Thus, it is not clear the degree to which the prolongation of signaling seen with the Y-577 mutant GHR was explained by a lack of SHP-2-mediated dephosphorylation of the receptor vs. diminished GH-induced receptor degradation.

Another SH2-containing protein that binds to the tyrosine phosphorylated GHR is the SOCS family protein CIS (cytokine inducible SH2 domain-containing protein) (53, 54). Recent work suggests that CIS, by virtue of its interaction with the GHR, promotes receptor internalization in response to GH and thereby contributes to desensitization of GH signaling (56). The contribution of CIS to GH-induced GHR degradation per se (as opposed to internalization) has yet to be directly studied. Interestingly, CIS, like other SOCS proteins, is likely linked to E3 ubiquitin ligase activity, which may serve to recruit proteins to the proteasome for degradation (57).

Recently, another E3 ubiquitin ligase, β-TrCP (β-transducin repeats-containing protein), has been implicated in the ligand-induced ubiquitination and proteolysis of two cytokine receptor family members, the interferon-α receptor 1 (IFNAR1) and the prolactin receptor (PRLR) (58-61). Those studies suggest that phosphorylation of specific serine residues within the cytoplasmic domain of IFNAR1 and PRLR are critical for ligand-induced association with β-TrCP and subsequent receptor ubiquitination and downregulation (59, 61). Intriguingly, catalytic activity of TYK2 is required for IFNAR1 ubiquitination because it was essential for phosphorylating IFNAR1 within the β-TrCP-binding motif (62). Furthermore, it has been reported that JAK2 catalytic activity is essential for the ubiquitination of erythropoietin receptor (EpoR) (63). While a role for β-TrCP in GH-induced GHR downregulation and in erythropoietin-induced EpoR downregulation has not been definitively found, it is notable that both GHR and EpoR contain a similar cytoplasmic domain sequence (DSG and surrounding amino acids) as that found in the PRLR and IFNAR1 to mediate phosphoserine-dependent recruitment of β-TrCP (61). Our results with the MYFc8 mutant make us consider whether β-TrCP

might affect GHR downregulation and, if so, whether receptor tyrosine phosphorylation, by impacting serine phosphorylation, could allow GHR-β-TrCP interaction. Future studies will be required to discriminate which, if any, of these mechanisms are at play.

The potential impact of receptor ubiquitination and specific ubiqutin ligases in regulation of GH-induced GHR downregulation and the effects of proteasome inhibitors on this process also raise the issue of the proteasome's involvement in these processes. Our work suggests that the proteasome is likely directly involved in degrading the GHR or its fragments under certain circumstances. We previously showed, for instance, that the soluble cytoplasmic domain of the receptor that results from intramembraneous cleavage by gamma secretase is degraded in a proteasome-dependent fashion (64). Recent work also suggests that in the absence of JAK2, newly synthesized GHR is degraded in an ERAD-like fashion and that this is blocked by proteasome inhibitors (Loesch, et al, submitted). While it is possible that the proteasome itself is involved in GH-induced GHR downregulation, we cannot rule out an indirect effect of proteasome inhibitors on this process because proteasome inhibition might suppress the lysosomal pathway via depletion of the ubiquitin pool (65). Future studies will address this important mechanistic issue and explore the ligases involved in GHR ubiquitination.

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SUMMARY

The accurate regulation of the magnitude and duration of signaling transduction is important and is required for normal life. Growth hormone (GH) signaling occurs when GH binding to growth hormone receptor (GHR), a member in type I cytokine receptor family, activates Janus kinase 2 (JAK2) that in turn phosphorylates GHR, followed by the activation of MAPK, PI3K and STAT5 pathways. The level of GHR at the cell surface is likely a key determinant of GH responsiveness and it is stabilized by JAK2. Our recent study indicated that JAK2 can stabilize GHR by physically associating with it. In the meantime, GH stimulation can downregulate GHR through JAK2. Apart from physical association, this GH-dependent GHR loss requires JAK2 kinase activity and GHR tyrosine residues. In the current study, we aimed to systematically delineate the mechanisms by which GH downregulates GHR. We found that mutation of tyrosine residues in the cytoplasmic domain of GHR reduced the internalization rate of GHR. However, mutation of the serine residues in the β-TrCP binding consensus motif of GHR did not disrupt GH-dependent GHR downregulation.

INTRODUCTION

Growth Hormone (GH), a ~22KD peptide, is a key regulator of postnatal growth and metabolism. It is primarily secreted by anterior pituitary gland in a pulsatile manner

and its synthesis is regulated by growth hormone releasing hormone (GHRH) and somatomedin (SMS). The secreted GH binds to its cognate receptor, growth hormone receptor (GHR). GHR, a transmembrane glycoprotein, belongs to the cytokine receptor superfamily based on its structure and it is synthesized in endoplasmic reticulum and undergoes glycosylation during the process to the cell surface through the Golgi. The receptor on the cell surface transduces signals in response to GH by the activation of the non-receptor tyrosine kinase JAK2 followed by activation of the STAT, MAP kinase and PI3K pathways.

Ligand depedent regulation of the receptor could affect the activities of the cell surface-associated as well as the intracellular receptors. Epidermal growth factor receptor (EGFR) down-regulation by its ligand is probably the best understood among growth factor receptors in mammalian cells (1). For GH receptor, a reduction of GH binding sites at the cell surface was shown in COS-7 cells pre-exposed to GH (2). Our previous study also provided evidence that GH can downregulate receptor (3). We further demonstrated that either JAK2 kinase deficiency or tyrosine mutation of GHR prevents GH-dependent GHR degradation (3). However, the mechanisms by which JAK2 kinase activity and GHR tyrosine phosphorylation regulate GH-dependent GHR loss are still unknown.

After reaching the cell surface, many plasma membrane proteins are endocytosed and ultimately degraded in the lysosome. Like many other membrane proteins, GH receptor has been shown to undergo clathrin-dependent endocytosis by immunoelectron

microscopy (4) and the GH-dependent internalization of GHR requires ubiquitin-dependent endocytosis (UbE) motif (5). Electron microscopy also showed that GHR located in lysosome (6). Since mutation of tyrosine residues of GHR prevents GH-induced receptor degradation, we asked whether tyrosine mutation of the receptor affects the trafficking of GHR from the cell surface to lysosome, including internalization, intracellular trafficking and recycling.

The process of receptor endocytosis is not exclusively regulated by the linear motifs located on the cytoplasmic tail of the receptors. Posttranslational modification by ubiquitination has also emerged as an important factor in the endocytosis and sorting of surface receptors. Whereas the polyubiquitination of proteins leads to their degradation by 26S proteasome complexes, the oligoubiquitination of plasma membrane proteins targets them for endocytosis and degradation in lysosome (7, 8). Three enzymes: ubiquitin-activating enzyme (E1) (9, 10), ubiquitin-conjugating enzyme (E2) (11, 12) and ubiquitin-protein ligase (E3) (13) are involved in the ubiquitination modification of proteins. The fate of protein substrates depends on E3 ligases that recognize the specific substrates and mediate their ubiquitination. SCF E3 ligase complexes are the largest family of E3s known (14). β-TrCP, which belongs to F box protein, is the substrate binding subunit of $SCF^{\beta-TrCP}$ E3 ligase (14). It recognizes and bind to phospho-motifs ($D_pSGX_{(n+2)p}S$) of the substrates (15). By analysis of amino acid sequence of growth hormone among different species, we found the existence of β-TrCP binding consensus motif (DSGRT/AS) in the cytoplasmic domain of GH receptor.

Ligand-induced GHR ubiquitination has been demonstrated in different cell systems (3, 16-18). Our previous study further showed that either JAK2 kinase activity deficiency or mutation of tyrosine residues of GHR abolished GH-induced GH receptor ubqituination, correlated with the dramatic decrease of GH-dependent GHR loss (3). Here we aimed to elucidate whether β-TrCP is involved in GH-dependent receptor ubiquitination and degradation by mutating the serine residues of the βTrCP recognition motif in GHR to alanine.

Mass spectrometry has been used to discover proteins or protein modification in various aspects (19-21). Apart from proteins that participating in ubiquitination, many other proteins are also involved in degradation of surface receptors. Cytokine-induced SH2 domain-containing protein CIS has been demonstrated to be implicated in growth hormone receptor internalization (22). Since the deficiency of JAK2 kinase activity or mutation of tyrosine residues of GHR markedly diminished GH-induced receptor downregulation, we hypothsized the proteins differentially associated with GHR in these conditions compared to wild-type JAK2 and wild-type GHR expressing cells. By using our established stable cell lines, we tended to identify these proteins by mass spectrometry.

MATERIALS AND METHODS

Materials

Recombinant hGH was kindly provided by Eli Lilly Co.. Fetal bovine serum,

gentamicin sulfate, penicillin, and streptomycin were purchased from Biofluids. Zeocin was purchased from Invitrogen. G418 and hygromycin were purchased from Mediatech Inc.. EZ-Link-SulfoNHS-S-S-Biotin and neutrAvidin beads were from Pierce Inc.. Iodoacetamide and sodium 2-mecaptoethanesulfonate were purchased from Sigma-Aldrich Inc. Other routine chemicals were bought from Fisher Scientific Inc.

Antibodies

Anti-$GHR_{cyt\text{-}AL47}$ polyclonal serum and the rabbit polyclonal antiserum, anti-$JAK2_{AL33}$ polyclonal serum has been described previously (3). The rabbit polyclonal anti-phosphorylated JAK2 was purchased from Upstate Biotechnology, Inc.. Monoclonal antiphosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology, Inc.. Anti-$GHR_{cyt\text{-}mAb}$ is a mouse monoclonal antibody ($IgG2b_{\kappa}$) directed against a bacterially expressed GST fusion protein incorporating hGHR residues 271–620 and has been previously described (23). Anti-$GHR_{ext\text{-}mAb}$, a mouse monoclonal antibody ($IgG1_{\kappa}$) directed against a bacterially expressed GST fusion protein incorporating rabbit GHR residues 1–246, has been previously described (23-25). Polyclonal anti-STAT5 was purchased from Santa Cruz Biotechnology, Inc.. Anti-phosphorylated STAT5 polyclonal antibody was purchased from Zymed Labratories, Inc.

Plasmids construction

The pSX-rbGHR was described previously (25). The pSX-rbGHR$_{S366A}$ and pSX-rbGHR$_{S366A/S370A}$ plasmids were constructed based on the parental plasmid pSX-rbGHR according to the instruction of QuikChange® Site-Directed Mutagenesis Kit from Stratagene Inc..

Cells, cell culture, and transfection

γ2A is a JAK2-deficient human fibrosarcoma cell line kindly provided by Dr. G. Stark (26). Stable transfection and maintenance of cell lines γ2A-JAK2, γ2A-GHR-JAK2, γ2A-GHR-JAK2$_{KD}$, γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 were described previously (3, 27, 28). Stable γ2A-GHR$_{S366A}$-JAK2 and γ2A-GHR$_{S366A/S370A}$-JAK2 cell lines were achieved by co-transfecting pSX-hygromycin-hemagglutinin, which encodes hygromycin resistance, and pSX-rbGHR$_{S366A}$ or pSX-rbGHR$_{S366A/S370A}$ plasmids respectively into γ2A-JAK2 cells individually using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Cells were selected in DMEM γ2A-JAK2 growth medium supplemented with 200ug/ml hygromycin and screened after 3-5 weeks for GHR expression by blotting with anti-GHR$_{AL-47.}$ These two stable cell lines were maintained in the same media as used for γ2A-GHR-JAK2.

Cell stimulation, protein extraction, electrophoresis, immunoprecipitation, and immunoblotting

Serum starvation of cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V, Roche Molecular Biochemicals) for serum in their respective culture media for 12-16 hrs prior to experiments. Cells were stimulated with hGH (500 ng/ml) in Dulbecco's modified Eagle's medium (1 g/L glucose) with 0.5% (w/v) bovine serum albumin at 37 °C. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) in the presence of 0.4 mM sodium orthovanadate (PBS-vanadate) to terminate the stimulation. Cells were solubilized in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine and 10 μg/ml aprotinin) on ice for 30-60 min. After centrifugation at 15,000 × g for 15 min at 4 °C, the detergent extracts were collected and electrophoresed by addition of Laemmli SDS-PAGE sample buffer.

For immunoprecipitation, cell extracts were mixed with appropriate antibody and incubated at 4 C overnight with continuous agitation. Conjugated Sepharose (Amersham Pharmacia Biotech) was added and incubated at 4 C for additional 2 h. The beads were washed four times with lysis buffer. Laemmli sample buffer eluates were resolved by SDS-PAGE and immunoblotted.

Resolution of proteins by SDS-PAGE, Western transfer of proteins, and blocking of Hybond-ECL (Amersham Biosciences) with 2% bovine serum albumin were

performed as previously described. Immunoblotting with anti-$GHR_{cyt\text{-}AL47}$ (1:4000), , anti-p-JAK2 (1:1000), anti-p-STAT5 (1:1000), anti-JAK2AL33 (1:4000), anti-STAT5 (1:1000) or 4G10 (1:1000), with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:10000) and ECL detection reagents (SuperSignal West Pico chemiluminescent substrate; all from Pierce Chemical Inc.) and stripping and reprobing of blots were accomplished according to the manufacturer's suggestions.

Surface biotinylation-based internalization assay

Serum-starved 100% confluent-cells were washed with ice-cold phosphate buffered saline (PBS) and kept in 4℃ for 10-15 min. Then cells were incubated with 0.375mg/ml EZ-Link-SulfoNHS-S-S-Biotin (Pierce Chemical Inc.) in 150mM $Na_2B_4O_7$ (PH=8.0) for 30min at 4℃. After binding, DMEM with 0.1% BSA was used to quench the excessive biotin. Then cells were either kept on ice (time point '0' and 100%-dish control) or incubated with 500ng/ml GH for 2 min, 7 min, or 15 min in 37℃ water bath. Cells, except 100%-dish, were subjected to the removal of the surface-bound biotin by sodium 2-mecaptoethanesulfonate (MESNA) treatment. All cells were lysed and biotinylated proteins were recovered by incubating with NeutrAvidin beads (Pierce Chemical Inc.) overnight, washed and subjected to immunoblotting with anti-$GHR_{cyt\text{-}AL47}$ antibody.

Densitometric and statistical analysis

Immunoblots were scanned using a high-resolution scanner (Hewlett-Packard Co.). Densitometric quantification of images exposed in the linear range was performed using an image analysis program, Image J (developed by W. S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data of densitometry from several experiments are displayed as mean ± SE.

RESULTS

Growth hormone receptor tyrosine phosphorylation enhances ligand-induced internalization.

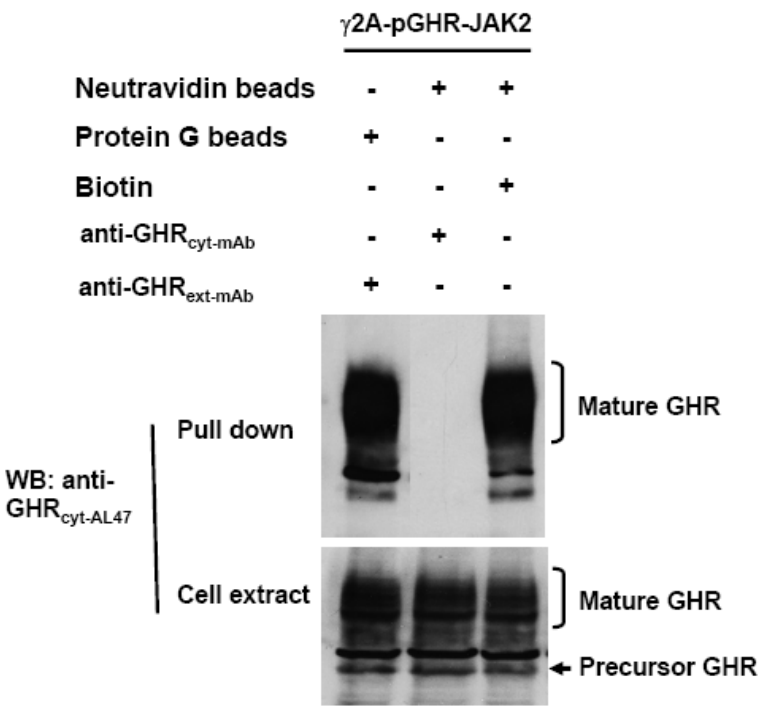
γ2A-JAK2 is a stable cell line which was made by stably transfecting γ2A, a JAK2- and GHR- deficient human fibrosarcoma cell, with JAK2 (27). We previously demonstrated that tyrosine phosphorylation of GHR is required for GH-dependent GHR downregulation by comparing γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 stable cell lines which were established by introducing either wild-type or tyrosine-mutant porcine GHR into γ2A-JAK2 cell (3). We sought to investigate whether the tyrosine mutation affected the receptor trafficking or not.

To determine the role of tyrosine phosphorylation on GHR endocytosis, we used the MYFc8 porcine GHR in which all eight cytoplasmic tyrosine residues were changed to phenylalanine v.s. wild-type porcine GHR (pGHR) (29). The GH-induced internalization rates of the receptors were compared between γ2A-pGHR-JAK2 and

Figure 1, Biotin binding to GHR did not affect GH-induced GH signaling.

A, Serum-starved γ2A-pGHR-JAK2 cells were incubated with anti-$GHR_{cyt\text{-}mAb}$ or anti-$GHR_{ext\text{-}mAb}$ for 1 hr or EZ-Link-SulfoNHS-S-S-Biotin on the shaker in cold room for 30 min. Cells were lysed after unbound antibodies or biotin was removed by ice-cold PBS. Detergent extracts were incubated with protein G beads or neutravidin beads for 1 hr to recover the antibody- or biotin- bound proteins. Eluates or cell lysates were resolved by SDS-PAGE and immunoblotted with anti-$GHR_{cyt\text{-}AL47}$ antibody. B, Serum-starved γ2A-pGHR-JAK2 cells were incubated with EZ-Link-SulfoNHS-S-S-Biotin or vehicle for 30 min in cold room followed by GH or treatment for 15 min. Detergent extracts were resolved by SDS-PAGE and sequentially blotted with anti-p-JAK2 and $JAK2_{AL33}$ or anti-p-STAT5 and STAT5.

A.


γ2A-pGHR-JAK2
Neutravidin beads - + +
Protein G beads + - -
Biotin - - +
anti-GHR cyt-mAb - + -
anti-GHR ext-mAb + - -
WB: anti-GHR cyt-AL47
Pull down
Mature GHR
Cell extract
Mature GHR
Precursor GHR

B.

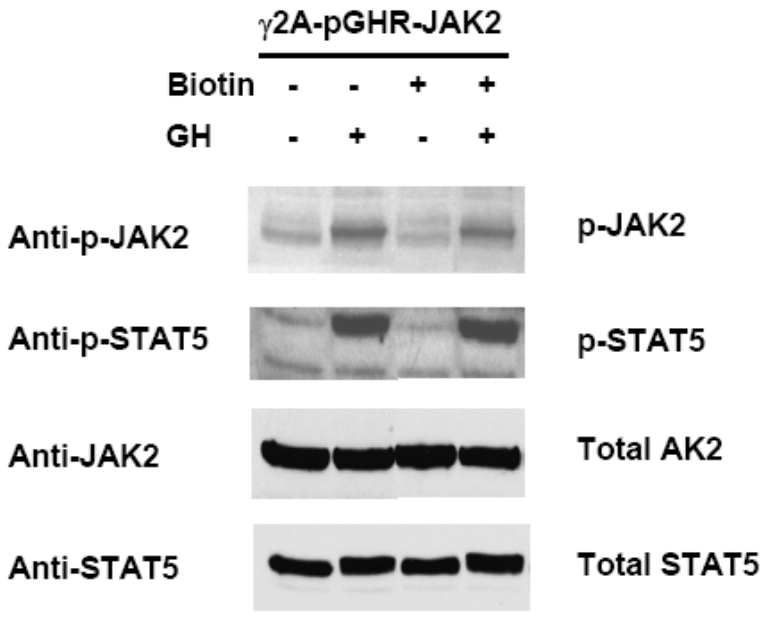
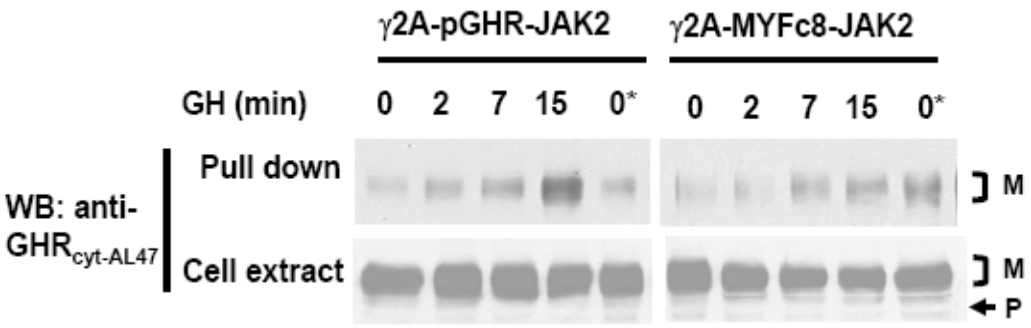

γ2A-pGHR-JAK2
Biotin - - + +
GH - + - +
Anti-p-JAK2
p-JAK2
Anti-p-STAT5
p-STAT5
Anti-JAK2
Total AK2
Anti-STAT5
Total STAT5

Figure 2, Mutation of tyrosine residues of GHR diminishes GH-induced GHR endocytosis.

A, Internalization of GHR was measured by reversible surface biotinylation assay. Birefly, serum-starved γ2A-pGHR-JAK2 or γ2A-MYFc8-JAK2 cells were incubated with EZ-Link-SulfoNHS-S-S-Biotin on the shaker in cold room for 30 min followed by GH treatment for 0-15min. Biotin on the cell surface was removed by MESNA. Then cells were lysed and cell extracts were incubated with neutravidin beads. Eluates or cell lysates were analyzd by immunoblotting using anti-$GHR_{cyt\text{-}AL47}$ antibody. '0*' denotes 10% of the samples that did not undergo biotin removal. B, Densitometric quantitation of anti-GHR blots from pull down assay, including those in A. Ten fold of the mature GHR at 0* was considered 100% for each experiment (mean ± SE; n = 3 independent experiments, $*$ $p<0.05$).

A.


γ2A-pGHR-JAK2
γ2A-MYFc8-JAK2
GH (min)
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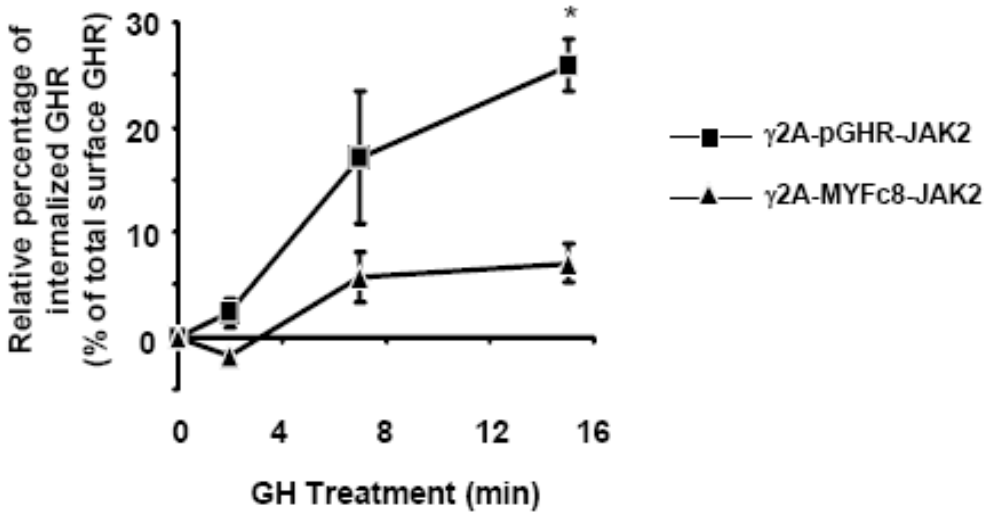
0 2 7 15 0*
WB: anti-GHRcyt-AL47
Pull down
Cell extract
M
M
P

B.


Relative percentage of internalized GHR (% of total surface GHR)
30
20
10
0
*
0 4 8 12 16
GH Treatment (min)
γ2A-pGHR-JAK2
γ2A-MYFc8-JAK2

γ2A-MYFc8-JAK2 stable cell lines by using reversible surface biotinylation assay. Briefly, γ2A-pGHR-JAK2 cells were incubated with biotin in cold room and cell lysates were incubated with neutravidin beads to pull down the surface GHR. A control experiment by using the antibody against extracellular part of GHR to pull down surface GHR as described previously was used to determine the efficiency of reversible surface biotinylation assay (30). Like antibody against extracellular part of GHR, the neutravidin beads pulled down surface GHR efficiently (Figure 1A). Also, in order to determine whether the biotin binding to GHR on the surface disrupts the binding of GH to GHR and GH signaling was examined. γ2A-pGHR-JAK2 cells were pre-incubated with biotin for 30 min at 4 C, followed by GH stimulation. GH treatment alone leaded to JAK2 and STAT5 phosphorylation as we observed before (3). Biotin pretreatment did not block GH-induced the phosphorylation of JAK2 and STAT5 (Figure 1B). This indicates that the biotin binding to GHR on the cell surface did not alter the binding of GH or GH signaling and the system can be used to track the fate of GH receptor in the presence GH. To measure the internalization rate of GHR by the system, γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells were pre-incubated with biotin at 4 C, followed by GH treatment in 37 C water bath for different time period. Surface GHR was removed by MESNA and internalized GHR was recovered by incubating cell lysates with neutravidin beads. About 25% of wild-type GHR was internalized at 15 min of GH treatment (Figure 2). Although tyrosine-mutant GHR still internalized, the internalization rate decreased to less than 10% at 15 min of GH treatment when all

tyrosine residues of GHR were mutated to phenylalanine (Figure 2). This indicates that the tyrosine phosphorylation of GHR facilitates GH-induced receptor internalization.

Serine residues in beta-TrCP consensus binding site in GHR cytoplasmic domain are not required for GH-induced GHR degradation.

Since disruption of JAK2 kinase activity and mutation of all tyrosine residues of GHR abolished both the GH-induced receptor ubiquitination and downregulation (3), we explored the possible potential E3 ligase involved in GH-dependent GHR loss. Since the cytoplasmic domain of GHR harbors the consensus sequence, $DSGX_{(n+2)}S$, which is conserved among species (Figure 3A) and is the recognition motif of β-TrCPs, the substrate-recognizing F-box containing component of $SCF^{\beta\text{-}TrCP}$ E3 ligase which mediates ubiquitination and degradation (14, 31-36). Since the serine residues (particularly the first one) in the consensus motif are believed to play an important role in the recognition of substrates, we assessed the possible role of β-TrCPs in GHR downregulation in the presence of GH by mutating the serine residues in β-TrCP binding consensus motif of rabbit GHR (Figure 3B). To examine this, cell lines γ2A-rbGHR$_{S366A/A370A}$-JAK2 and γ2A-rbGHR$_{S366A}$-JAK2 which stably express wild-type JAK2 and the serine mutant rabbit GHR were established to be compared with γ2A-rbGHR-JAK2 stable cell which expresses wild-type JAK2 and wild-type rabbit GHR (28). Two individual stable clones for each serine-mutant GHR expressing stable cell lines were compared to wild-type GHR expressing cell (C14). The disulfide-linked GHR (dsl GHR) arises from covalent

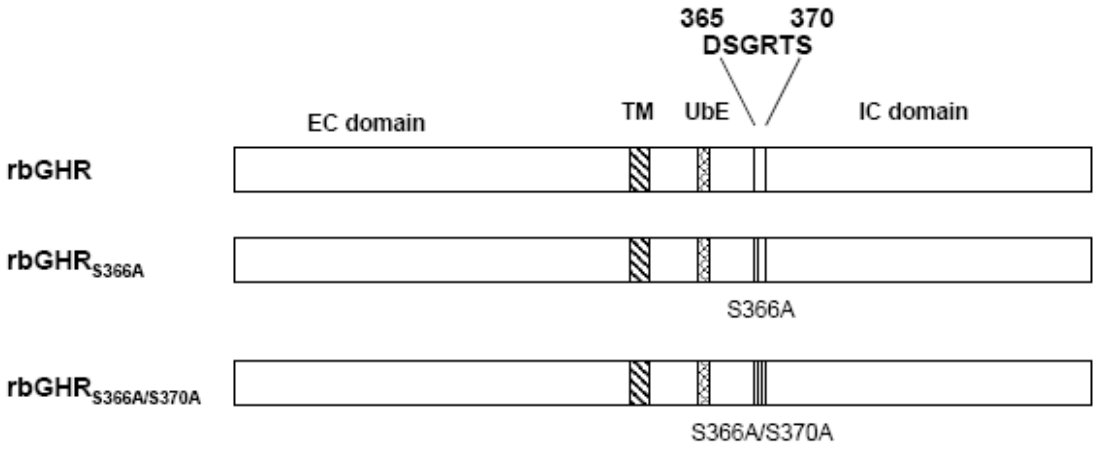
Figure 3, Mutation of serine residue(s) in DSGRTS motif of GHR does not affect GH-induced GHR disulfide linkage formation or GH signaling.

A, Sequence alignment of the β-TrCP consensus binding motif, DSGXXS, in the intracellular domain of GHR from different species. B, Structure of the rabbit GHR (rbGHR) constructs. GHR contains extracellular (EC) domain, transmembrane (TM) domain and intracellular (IC) domain. Ubiquitin-dependent endocytosis (UbE) motif and DSGRTS motif are in the cytoplasmic domain. Two rbGHR mutants, $rbGHR_{S366A}$ and $rbGHR_{S366A/S370A}$, contains serine to alanine mutation in DSGRTS motif respectively. C and D, The effect of serine mutation on GH-induced GHR disulfide linkage formation or GH-induced GH signaling. Serum-starved γ2A-JAK2 cells stably expressing WT or mutant GHR were stimulated with 500 ng/ml of GH or vehicle for 30 min. Cell extracts were solved by non-reducing (C) or reducing (D) gel and blotted with anti-$GHR_{cyt\text{-}AL47}$, or sequentially blotted with anti-p-JAK2 and anti-$JAK2_{AL33}$ or anti-p-STAT5 and anti-STAT5 respectively.

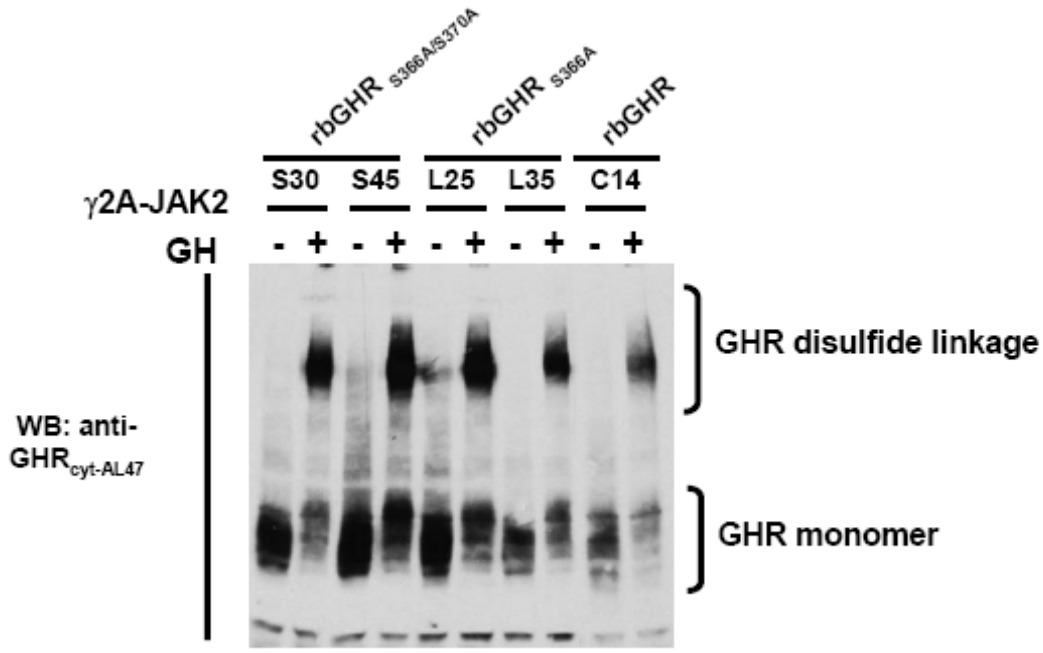
A.

--DSGRAS--	Turbot
--DSGRAS--	Xenopus
--DSGRAS--	Turtle
--DSGRAS--	Chicken
--DSGRTS--	Rat
--DSGRTS--	Rabbit
--DSGRTS--	Pig
--DSGRTS--	Human

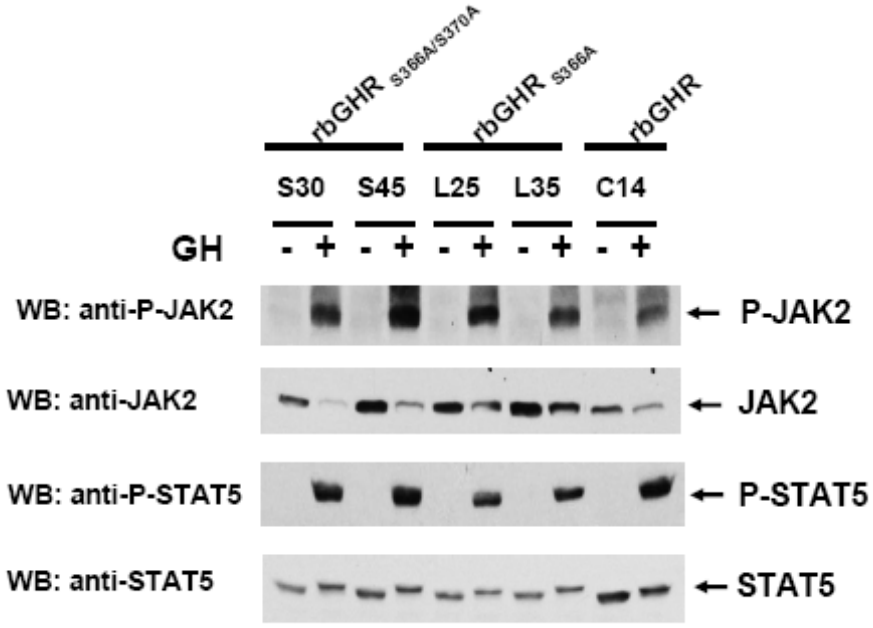
B.



C.


rbGHR S366A/S370A
rbGHR S366A
rbGHR
S30
S45
L25
L35
C14
γ2A-JAK2
GH
- + - + - + - + - +
WB: anti-GHR cyt-AL47
GHR disulfide linkage
GHR monomer

D.


rbGHR S366A/S370A
rbGHR S366A
rbGHR
S30
S45
L25
L35
C14
GH
- + - + - + - + - +
WB: anti-P-JAK2
P-JAK2
WB: anti-JAK2
JAK2
WB: anti-P-STAT5
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STAT5

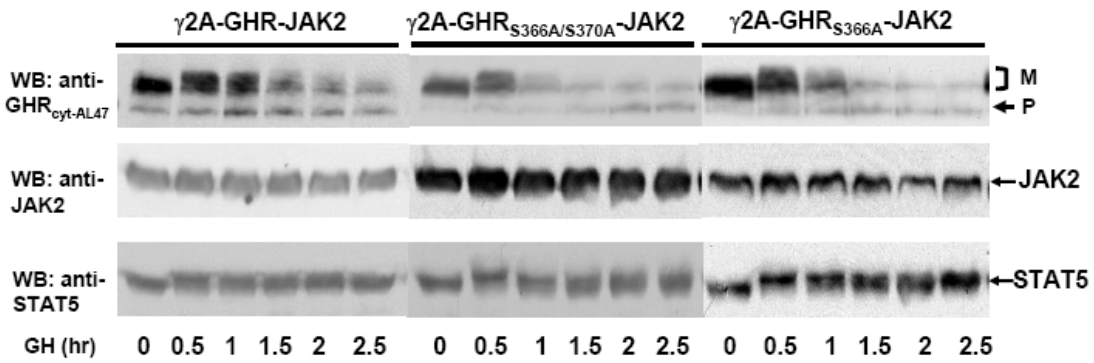


Figure 4, Mutation of serine residue(s) in DSGRTS motif of GHR does not affect GH-induced GHR downregulation.

Serum-starved γ2A-GHR-JAK2, γ2A-GHR$_{S366A/S370A}$-JAK2 and γ2A-GHR$_{S366A}$-JAK2 stable cells were treated with 500 ng/ml GH for 1-2.5 hr. Cell extracts were solved by SDS-PAGE and blotted with anti-GHR$_{cyt-AL47}$, anti-JAK2$_{AL33}$, and anti-STAT5.

bonding between receptors mediated by cysteine-241, the only unpaired extracellular cysteine and its appearance can be used to monitor the GHR's attainment of an activated conformation (23). As seen in Figure 3C, cells expressing serine mutant GHR responded to GH treatment with the formation of the disulfide-linked form of GHR. To assess downstream signaling, we examined the activation of JAK2 and STAT5. Serine mutation of GHR did not alter GH-induced the phosphorylation of JAK2 and STAT5 (Figure 3D). This suggests that mutation of the serine residues of GHR in β-TrCP binding consensus motif, DSGRTS, does not affect receptor engagement by GH or alter GH-induced GH signaling.

To seek evidence of the involvement of the serine residues of GHR in β-TrCP binding consensus domain in GH dependent receptor loss, GH receptor was tracked in both serine-mutant GHR stably expressing cells (γ2A-rbGHR$_{S366A/A370A}$-JAK2 and γ2A-rbGHR$_{S366A}$-JAK2) and wild-type GHR expressing cell (γ2A-rbGHR-JAK2). As we previously demonstrated, GH treatment caused dramatically wild-type mature GH receptor loss (Figure 4) (3). Unexpectedly, mutation of serine residue(s) of GHR in β-TrCP binding consensus motif, DSGRTS, did not decrease the degradation rate of GHR (Figure 4). This indicates that serine residues of GHR in β-TrCP binding consensus motif, DSGRTS, are not required for GH-induced receptor downregulation.

Preliminary data for exploring proteins which mediate GH-induced GHR degradation by discovery approach.

Another approach to explore the mechanism of GH-dependent receptor loss is to identify proteins implicated by mass spectrometry. Since the degradation rates are different between wild-type porcine GHR and tyrosine-mutant GHR, we tended to find proteins that differently associate with wild-type or mutant GHR in the presence of GH by immunoprecipitation followed by mass spectrometry.

In GH signaling, ligand binding to GHR causes conformational change of the dimerized receptors, leading to the activation of JAK2 and tyrosine phosphorylation of GHR. The association of activated JAK2 with tyrosine tyrosine phosphorylated GHR allows co-immunoprecipitation of phospho-JAK2 and phospho-GHR. To ascertain that our approach works, γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells were stimulated with GH for 2 min and cell lysates were incubated with anti-$GHR_{cyt\text{-}AL47}$ antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and blotted with anti-phosphotyrosine antibody (4G10) which detects tyrosine phosphorylated proteins. As we know, JAK2 can be phosphorylated in both γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells in the presence of GH, however GHR tyrosine phosphorylation can be detected only in γ2A-pGHR-JAK2 cell because all the tyrosine residues in the cytoplasmic domain of GHR were mutated to phenylalanine in γ2A-MYFc8-JAK2 cell (3, 29). Consistent with our previous study, GH receptor and JAK2 can be basally tyrosine phosphorylated and associated in γ2A-pGHR-JAK2 cell probably due to the

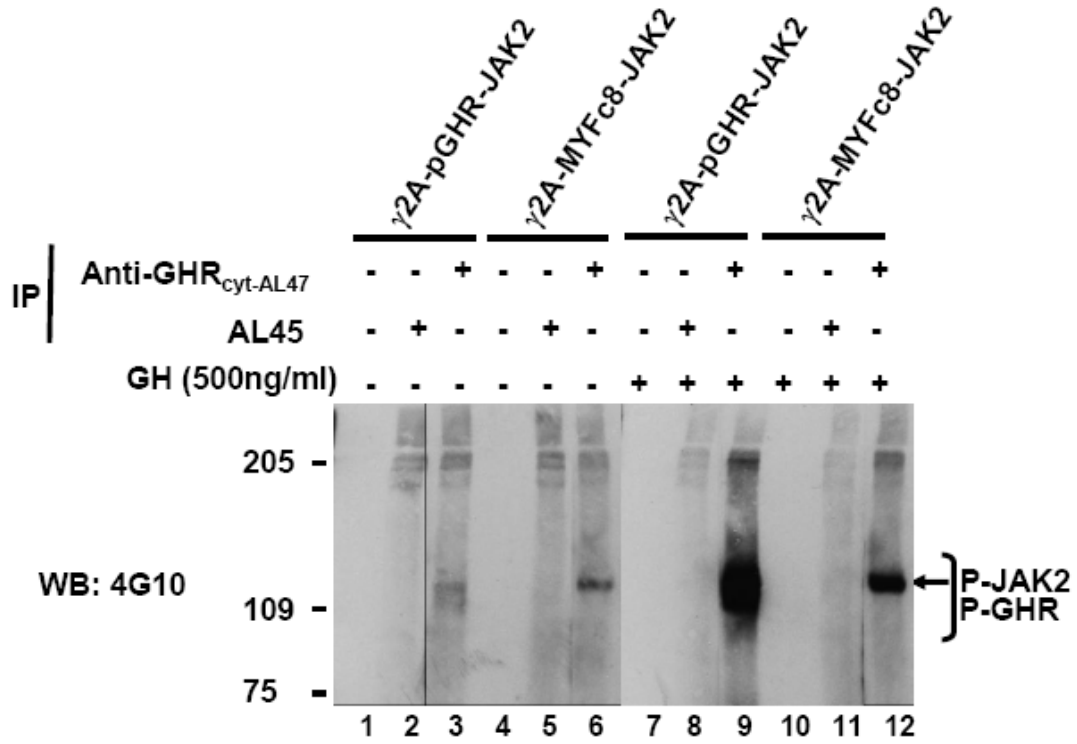


Figure 5, Phosphorylated proteins immunoprecipitated by anti-$GHR_{cyt\text{-}AL47}$.

Serum-starved γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 were treated with 500 ng/ml GH or vehicle for 7 min. Detergent (1% triton x-100) extracts were immunoprecipitated with anti-$GHR_{cyt\text{-}AL47}$ or control antibody AL-45. Eluates were solved by SDS-PAGE and blotted with 4G10.

overexpression of GHR and JAK2 in the cell (lane 3, Figure 5), and GH stimulation dramatically increased the recruitment of JAK2 followed by increase of tyrosine phosphorylation of JAK2 and GHR (lane 9, Figure 5); In contrast, a sharp band (phosphor-JAK2) was basally detected in γ2A-MYFc8-JAK2 cell and ligand treatment allows more JAK2 phosphorylated and associated with tyrosine mutant GHR (lane 6 and lane 12, Figure 5). This demonstrates that our immunoprecipitation approach can be used to detect proteins differentially associated with GH receptor.

Commassie blue staining will be done to identify interested proteins bands for mass spectrometry study. Apart from JAK2, a lot of other proteins (including non-tyrosine phosphorylated proteins) can interact with GHR, and can be pulled down by coimmunoprecipitation and theoretically identified by coomassie brilliant blue staining. Since anti-$GHR_{cyt\text{-}AL47}$ antibody is polyclonal antibody against cytoplasmic domain of GHR, it could recognize non-specific proteins which will make our work more complicated. To simplify the work, anti-$GHR_{cyt\text{-}mAb}$, a purified monoclonal antibody against the cytoplasmic domain of GHR, was used to be compared with anti-$GHR_{cyt\text{-}AL47}$ to verify that the monoclonal antibody can also pull down GHR and associated JAK2. Also, in order to precipitate proteins that weakly associate with GHR, low percentage of triton X-100 (0.15%) containing lysis buffer, in contrast to routinely used 1% triton x-100 containing buffer, was used to lyse cells. γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells were stimulated with GH for 2 or 5 min. As we saw in Figure 5, immunoprecipitates from anti-$GHR_{cyt\text{-}mAb}$ also included basally phosphorylated

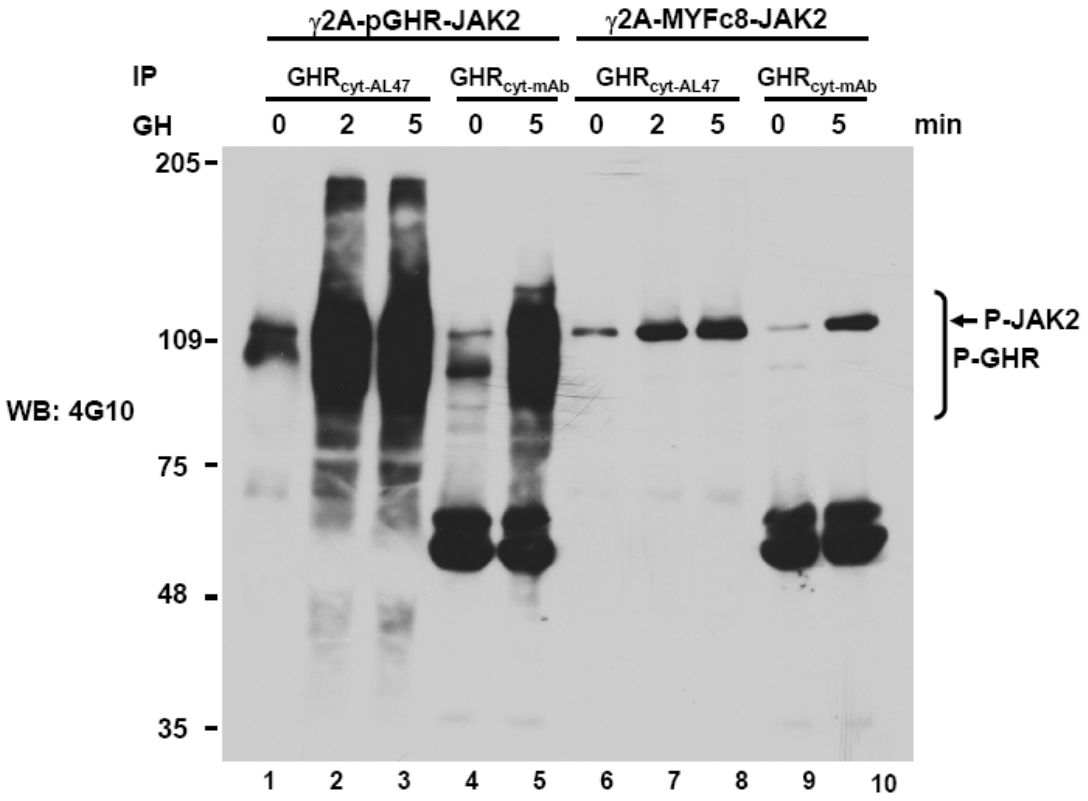


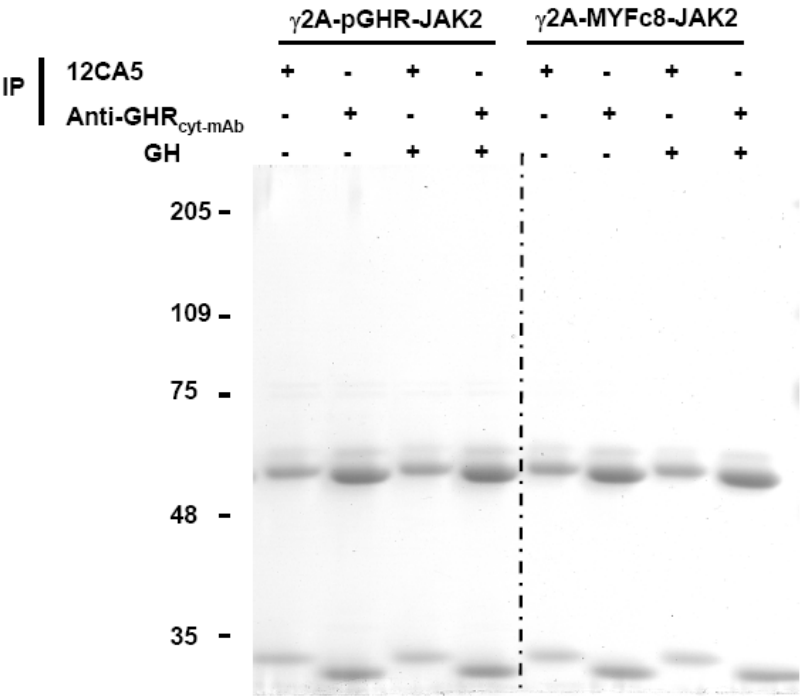
Figure 6, Comparison of phosphorylated proteins immunoprecipitated by anti-$GHR_{cyt\text{-}AL47}$ and anti-$GHR_{cyt\text{-}mAb}$.

Serum-starved γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 were treated with 500 ng/ml GH or vehicle for 2 or 5 min. Detergent (0.15% triton x-100) extracts were immunoprecipitated with anti-$GHR_{cyt\text{-}AL47}$ or anti-$GHR_{cyt\text{-}mAb}$. Eluates were solved by SDS-PAGE and blotted with 4G10.

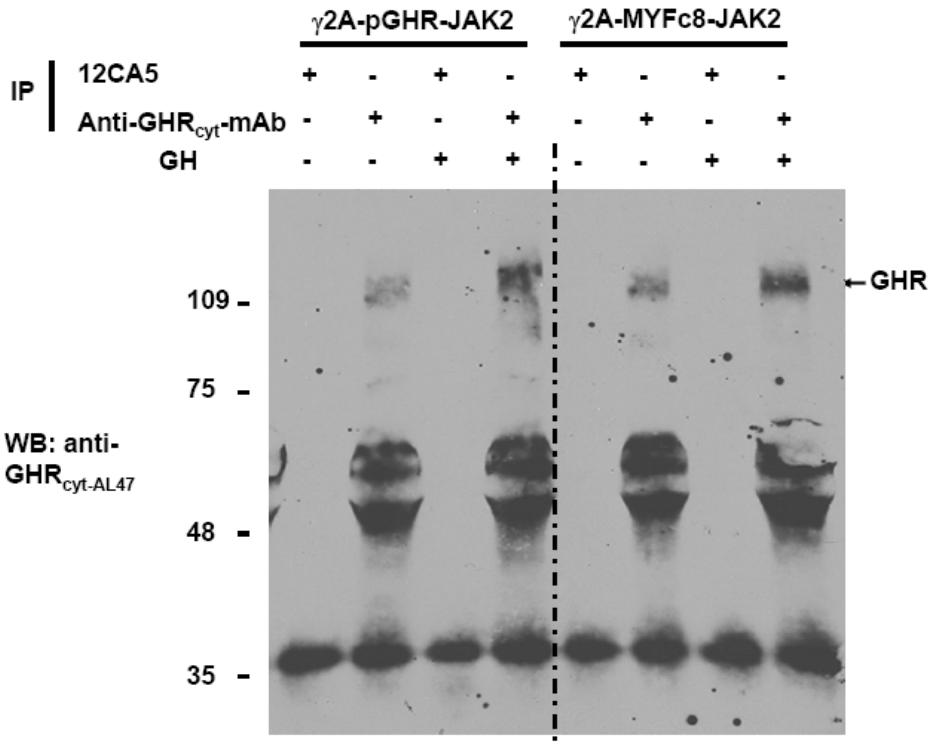
Figure 7, Coomassie brilliant blue staining of total proteins immunoprecipitated by anti-$GHR_{cyt\text{-}mAb}$.

Serum-starved γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 were treated with 500 ng/ml GH or vehicle for 2 min. Detergent (0.15% triton x-100) extracts were immunoprecipitated with anti-$GHR_{cyt\text{-}mAb}$. Eluates were solved by SDS-PAGE and stained by coomassie brilliant blue (A) followed by western blotted with anti-$GHR_{cyt\text{-}AL47}$ (B).

A.


γ2A-pGHR-JAK2
γ2A-MYFc8-JAK2
IP
12CA5
Anti-GHR$_{cyt-mAb}$
GH
205
109
75
48
35

B.


γ2A-pGHR-JAK2
γ2A-MYFc8-JAK2
IP
12CA5
Anti-GHR$_{cyt}$-mAb
GH
109
75
48
35
WB: anti-GHR$_{cyt-AL47}$
GHR

JAK2 (lane 4, Figure 6) and ligand stimulation markedly increased the amount of precipitated phosphorylated proteins including GHR (bracket) and JAK2 (arrow) in γ2A-pGHR-JAK2 cell (lane 3 v.s. lane 5, Figure 6) while only phosphorylated JAK2 can be detected in the precipitates in γ2A-MYFc8-JAK2 cell and the amount of phosphorylated JAK2 greatly enhanced when cell was stimulate with GH (lane 9 and lane 10, Figure 6). This suggests that anti-$GHR_{-cyt\text{-}mAb}$ can be employed to immunoprecipitate GHR-associated proteins.

Our aim is to discover proteins that differently associate with wild-type GHR and tyrosine-mutant GHR when cells are treated with GH. As we mentioned, coomassie brilliant blue staining will be used to compare the protein profiles to discover interested proteins in γ2A-pGHR-JAK2 and γ2A-MYFc8-JAK2 cells. After GH treatment, cell lysates were incubated with GHR monoclonal antibody, anti-$GHR_{-cyt\text{-}mAb}$. Immunoprecipitates were resolved by SDS-PAGE followed by coomassie brilliant blue staining. Anti-HA monoclonal antibody (12CA5) was used as control. Coomassie brilliant blue staining did not detect other proteins including JAK2 except light chain and heavy chain of immunoglobin (Figure 7A). The absence of JAK2 in GHR precipitates even in the presence of GH treatment indicates the coomassie brilliant blue staining was not sensitive enough for visualization of the low amount of coimmunoprecipitated proteins even though western blot showed their presence (Figure 7B).

CONCLUSIONS AND DISCUSSION

In this study, we attempted to investigate the mechanism(s) by which GH induced GH receptor degradation. After reaching the cell surface, some mature GH receptor endocytoses into early endosome, then trafficks to late endosome and eventually degrades in lysosome (4, 6, 37, 38). Since the fates of GHR are quite different between wild-type GHR expressing cell and tyrosine-mutant GHR expressing cell as shown by our previous study, the trafficking of growth hormone receptor was examined in the presence of GH. The first step, internalization, of the trafficking of cell surface GHR was compared in these two cells first in this study. By labeling cell surface protein using biotin and tracking the internalized biotin-labeled protein using neutravidin beads, we found that GH receptor internalized fast in respond to GH treatment. Mutation of all tyrosine residues in the cytoplasmic domain of GHR greatly decreased its internalization rate. Intracellular trafficking of GH receptor, including localization of GHR in late endosome and lysosome, and recycling needs to be examined to investigate the possible mechanisms involved. Since JAK2 kinase activity deficiency also abolishes the GH-depedent receptor loss, the question of whether the deficiency of JAK2 kinase activity also impairs the trafficking of GHR can be sovled by comparing wild-type JAK2 expressing cell (γ2A-rbGHR-JAK2) and kinase-deficient JAK2 expressing cell (γ2A-rbGHR-JAK2$_{KD}$).

Ubiquitin-dependent degradation of receptors is emerging as a key mechanism that regulates the magnitude and duration of signaling and its effects on cells and tissues

(39). Ubiquitination of GH receptor was noted in early purification study (16) and different cell systems (3, 17, 18). GH-dependent receptor ubiquitination was largely diminished by JAK2 kinase deficiency or GHR tyrosine mutation, as correlated with GH-induced GHR loss (3). However, the mechanisms of how ubiquitination of its cognate receptor for growth hormone mediates receptor degradation are yet to be elucidated. β-TrCP, the substrate recognition subunit of $SCF^{\beta TrCP}$ E3 ligase, has been shown to mediate the ubiquitination and degradation of proteins with serine phosphorylated within consensus motif, $D_pSGX_{(n+2)p}S$ (15). The phosphorylation of the serine residue(s), especially the first one, is required for the interaction of β-TrCP and its substrates (39, 40). The consensus motif is also highly conserved in the cytoplasmic domain of GHR among species. Our study indicated that mutation of both serine residues in the β-TrCP binding construction motif of rabbit GHR did not change the fate of GH receptor. This was confirmed by the recent publication from Dr. Strous' laboratory (41). However, β-TrCP does affect GHR endocytosis and abundance (41). In contrast to routine consensus β-TrCP binding motif, DSGXXS, the same group showed that β-TrCP bound to the ubiquitin-dependent endocytosis (UbE) motif of GHR using truncated GHR by *in vitro*-binding assay (41). Since this was done using truncated GHR *in vitro*, it may not represent *in vivo* physical status and needs to be further confirmed *in vivo*.

Other than β-TrCP and cytokine-induced SH2 domain-containing protein CIS, many other proteins might be involved in restrict the magnitude and duration of GH

signaling by regulating the abundance of GHR. These proteins can be unveiled by isolating proteins from the immunopreciptated complex by GHR antibody followed by mass spectrometry, which method is also used to uncover the implication of AP-2 protein complex in prolactin recptor (PRLR) degradation (19). Preliminary experiments were done to make sure that the antibodies including anti-$GHR_{cyt\text{-}AL47}$ and anti-$GHR_{cyt\text{-}mAb}$ used for immunoprecipitation work. Both antibodies can efficiently pull down phosphorylated GHR and JAK2 in wild-type porcine GHR expressing cell, while phosphor-JAK2 only in tyrosine-mutant GHR (MYFc8) expressing cell as indicated by anti-phosphotyrosine antibody (4G10) blot. Since anti-$GHR_{cyt\text{-}mAb}$ is purified monoclonal antibody and it recognizes GHR and pull down GHR-associated proteins more specifically, this antibody was later used to pull down GHR complexes. However, our pilot experiments with expanded cell culture did not allow us to see any other proteins including GHR other than immunoglobulin in coomassie blue staining although GHR was observed by western blot. This indicated that a more effective staining or more cultured cells are required for us to get the band from gel for analysis by mass spectrometry.

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SUMMARY

Growth hormone is a 22 kD polypeptide largely produced in the anterior pituitary gland (135). It has been known as a key regulator of postnatal growth and metabolism of carbohydrate, protein and lipid (20-23). GH is secreted in a pusatile pattern and the secretion of GH by the pituitary is regulated by the hypothalamus, where GHRH and somatostatin, respectively, provide positive and negative control of circulating GH levels (3, 136). The action of GH is initiated by binding to its cognate receptor, GHR, which is expressed in various target tissues. The GHR is a single membrane-spanning type Ⅰ glycoprotein of the cytokine receptor superfamily which includes receptors for prolactin (PRL), erythropoietin (EPO), and various interleukins among others. The GHR is a non-tyrosine kinase receptor and the association of cytoplasmic tyrosine kinase, JAK2, with the cytoplasmic domain of GHR is crucial for GH-induced signaling (96). JAK2, like other Janus kinases (JAKs 1-3, and Tyk2), has in its C-terminus a tyrosine kinase domain and a non-functional kinase-like domain. The interaction requires the membrane proximal region of GHR cytoplasmic domain called Box1 and N-terminus FERM domain of JAK2. One molecule of GH binding to the predimerized GHR at the cell surface triggers enhanced association with and activation of JAK2 (51), which then phosphorylates GHR, resulting in activation of MAPK, PI3K and STAT pathways.

GH sensitivity is largely determined by the abundance of GHR on cell surface GHR. Thus, it is critical to appreciate the factors governing GHR surface availability, the regulation of which can occur at the transcriptional, post-transcriptional, and post-translational levels. GHR gene expression and post-transcriptional mRNA processing are tightly regulated (84-86). Post-translational regulation of GHR cell surface abundance includes maturation, proteolytic shedding of GHR, constitutive and GH-induced GHR downregulation via endocytosis, and recycling.

GHR endocytosis has been extensively studied. It requires an intact ubiquitin-proteasome system and the ubiquitin-dependent endocytosis (UbE) motif in the membrane proximal region of GHR cytoplasmic domain, but not signal transduction via JAK2 (44-46, 48, 92, 93, 104, 117). There is little evidence about recycling of GHR to the cell surface up to now. One α-secretase, TACE, has been identified as a sheddase, which cleaves GHR at the site eight residues from the membrane in the proximal extracellular domain stem region to form soluble extracellular domain when cells were treated with phorbol myristate acetate (PMA), a protein kinase C activator, serum, or platelet-derived growth factor PDGF (98, 99).

JAKs are critical kinases for cytokine signaling transduction. In addition to mediating signaling, emerging evidence suggests JAKs influence surface levels of cytokine receptors with which they associate. Whether acting as chaperones for surface expression or negatively regulating constitutive downregulation of the receptors (106, 109-112), the mechanisms vary with each JAK. Our recent studies demonstrated that

nascent precursor GHR in cells lacking JAK2 undergoes endoplasmic reticulum-associated degradation (ERAD) and JAK2, by its association with GHR, rather than via its kinase activity, helps the dimerized precursor to avoid ER quality control (90). The association of JAK2 also increases susceptibility of GHR to inducible metalloprotease-mediated proteolysis and shedding of the extracellular domain (101). Like other cytokine receptors, JAK2 strongly affects the fate of mature surface GHR in γ2A system, a GHR- and JAK2- deficient human fibrosarcoma cell. Stable expression of JAK2 into γ2A-GHR stable cell line enhances the maturation of GHR and stabilizes cell surface GHR (53, 91). This dissertation continues to focus on the effects of JAK2 on surface GHR abundance: 1) How does JAK2 stabilize surface GHR? 2) Does JAK2-mediated GH signaling have any impact on the fate of GHR and, if so, how?

Recent studies found that JAKs alter the cellular distribution of their cognate cytokine receptor. To investigate how JAK2 stabilizes GHR on the cell surface, we first studied the role of GHR-JAK2 association in regulating surface stability of GHR by tracking the fate of mature GHR in the presence of cycloheximide (CHX), a new protein synthesis inhibitor. Our lab has demonstrated that the membrane proximal region of GHR called Box 1 element and N-terminus of JAK2 are required for GHR-JAK2 interaction therefore GH signaling (41, 58). By stably expressing either wild-type (WT) or mutant GHR into γ2A or γ2A-JAK2, and comparing the half-life of GHR in γ2A-GHR, γ2A-GHR$_{\Delta Box1}$, γ2A-GHR-JAK2 and γ2A-GHR$_{\Delta Box1}$-JAK2, we found that the half-life of mature GHR with Box 1 deleted is about 1.5 h, which was similar to that in γ2A-GHR

and was unaffected by expression of JAK2. In contrast, the half-life of wild-type GHR in JAK2-expressing cells was > 4 h. This indicates JAK2 can not stabilize mature GHR with the deletion of Box 1. Furthermore, reconstitution of γ2A-GHR with $JAK2_{\Delta 1–47}$, a mutant JAK2 lacking N-terminal 47 residues, did not alter the GHR stability. In contrast, another JAK2 mutant, $JAK2_{1–511\text{-}HA}$, which includes the first 511 residues and thus has the intact N-terminus but lacks the pseudokinase and kinase domains, can increase the mature GHR half life as WT JAK2 does. These data indicate that the JAK2-GHR association, which is mediated by the receptor Box 1 element and the JAK2 FERM domain, but not the pseudokinase and kinase domains of JAK2, is required for enhanced stability of the mature GHR. This is consistent with the observations for the effects of Tyk2 or JAK2 on the stability of IFNAR1 and TpoR and unlike the common γc, the enhanced accumulation of which by JAK3 requires the full-length, not just N-terminus of JAK3 (110-112).

We also studied the effects of JAK2-mediated GH signaling on the fate of GHR. Treatment of γ2A-GHR-JAK2 cells with CHX alone vs. CHX plus GH for 0-5 h revealed marked GH-induced GHR loss, with the half-life of the receptor reduced from > 4 h to ~ 1h. This important finding addressed the controversial question as to whether GH increases GHR downregulation. In contrast, GH had no effect on GHR loss in cells lacking JAK2. Thus, GH-induced GHR degradation depends on the presence of JAK2. In JAK2-replete cells, a GHR Box1 mutant did not undergo GH-dependent downregulation; nor did WT GHR when expressed with a FERM domain-disrupted

JAK2 ($JAK2_{\Delta 1-47}$). Notably, in contrast to its prevention of consitituve GHR downregulation, $JAK2_{1-511}$, which has an intact FERM domain, but no kinase-like or kinase domains, was unable to mediate GH-induced GHR loss. A kinase-deficient JAK2 mutant, $JAK2_{KD}$, which has point mutations at residues 1020 (W to G) and 1024 (E to A) that abrogate its phosphotransferase activity, failed to mediate GH-induced GHR degradation. Previously, Alves dos Santos and their colleagues compared the turnover of WT GHR and GHR_{4P-A}, a mutant GHR with the four proline residues between amino acids 280-287 in box-1 of the cytosolic tail mutated to alanines, therefore abrogate the association of JAK2 with GHR and GH-dependent activation of JAK2. They found that the GHR turnover was not affected by a mutated box-1 in the GHR and concluded that GH-induced GHR degradation was independent of JAK2 (117). Our elegant system allows us to pinpoint that not only the presence of JAK2 but also JAK2 kinase activity is required for GH-dependent GHR downregulation. In pharmacologic studies, both lysosome and proteasome inhibitors blocked GH-induced receptor loss, indicating that, like constitutive GHR downregulation, intact proteasome activity is needed for effective GH-induced degradation of GHR in lysosomes. However, whether proteasome inhibitor exerts its effect by depleting cellular ubiquitin by blocking of turnover of ubiquitinated proteins is not yet known.

We further pursued the GH-induced GHR loss using a pig (p) GHR mutant in which all the tyrosine residues in the cytoplasmic domain of the GHR were mutated to phenylalanines (MYFc8), and therefore, could not be tyrosine phosphorylated. When

coexpressed with JAK2, MYFc8 allowed GH-induced JAK2 tyrosine phosphorylation similar to that with WT pGHR, while GH-induced STAT5 activation was greatly reduced in MYFc8- vs. pGHR-expressing cells. Despite allowing GH-induced JAK2 activation, MYFc8 was only subtly downregulated in response to GH in comparison to pGHR. Noticeably, GH-induced GHR ubiquitination was also dramatically reduced in cells harboring MYFc8 and in cells coexpressing WT GHR with kinase-dificient JAK2. These data indicate that GH-induced GHR ubiquitination depends on both JAK2 kinase activity and GHR tyrosines or tyrosine phosphorylation. Importantly, in contrast to previous reports that the GH-induced endocytosis and degradation of GHR did not require the ubiquitination of GHR itself although an intact ubiquitin-conjugating system was necessary (44, 45, 104), our findings suggest that GHR ubiquitination per se might play a role in its downregulation.

We further explored the mechanism(s) by which JAK2 kinase activity or GHR tyrosine residues or tyrosine phosphorylation contributes to GH-induced GHR downregulation by several approaches. GH induces GHR downregulation through internalization, trafficking through intracellular vesicles and finally degradation in lysosome. The first step of GH-dependent GHR degradation, endocytosis, was examined by surface biotinylation-based internalization assay. Biotin bound surface GHR as well as the anti-GHR_{ext}-mAb, which is a mouse antibody against the extracellular domain of rabbit GHR. The binding of biotin to surface GHR did not affect GH-induced activation of JAK2 and phosphorylation of STAT5. GH induced

internalization of WT pGHR when coexpressed with JAK2 as reported (104). However, the internalization rate of mutant GHR (MYFc8) greatly reduced when the tyrosine residues in the cytoplasmic domain of GHR were mutated to phenylalanines. Further trafficking of GHR from early endosome to lysosome will be investigated. In the same token, whether the prevention of GH-induced GHR downregulation by JAK2 kinase deficiency is due to the decreased endocytosis or altered trafficking can be examined by compairing γ2A-GHR-JAK2 and γ2A-GHR-JAK2$_{KD}$ cells.

GHR ubiquitination was observed as early as the purification study (132). As with the prevention of GH-induced GHR downregulation, tyrosine mutation of GHR or JAK2 kinase deficiency also blocked GH-induced GHR ubiquitination. Until now, no identified E3 ligase for GHR has been reported. Comparison of alignment of GHR sequences from different species found a highly conserved sequence, DSGRA/TS, below the ubiquitin-dependent endocytosis (UbE) motif. This sequence is a consensus motif recognized by β-TrCP, the substrate recognition subunit of SCF$^{\beta TrCP}$ E3 ligase, which has been shown to mediate the ubiquitination and degradation of proteins with serine phosphorylation within consensus motif, D$_p$SGX$_{(n+2)p}$S (119). Unlike PRLR and IFNAR1 whose degradation are markedly diminished by mutation of serine residues in DSGX$_{(n+2)}$S in the receptors (50, 134), mutation of the two serine residues within the DSGRTS in the cytoplasmic domain of rabbit GHR did not prevent GH-induced GHR downregulation. However, we cannot exclude the possibility of the involvement of β-TrCP in GH-induced GHR loss. A research group recently reported that knockdown

of β-TrCP enhanced GHR expression and reduced its endocytosis thereby regulating its degradation (137). But an *in vitro*-binding assay demonstrated that β-TrCP bound to the UbE, not DSGRTS motif, of the truncated GHR (137). This observation is different from PRLR or IFNAR1, where the interaction of which with β-TrCP depdends on the $DSGX_{(n+2)}S$ motif (50, 134).

The cytokine-inducible SH2 domain-containing protein (CIS), a member of the COCS family, inhibits GHR signaling to STAT5b. It has been found to play an important role in regulating GHR internalization following receptor phosphorylation. Expression of a dominant-negative SH2 domain mutant CIS, CIS-R170K, inhibited the internalization of GH and GHR (105). However, whether the different fate of GHR between WT GHR and the tyrosine-mutant GHR (MYFc8) is due to the differential association of CIS is still unknown. Many other proteins other than β-TrCP and CIS might also be involved in the degradation of GHR. Discovery approaches can be used to identify these proteins.

Taken together, this dissertation furthers our understanding of mature GHR stability and sheds light on GH-induced GHR downregulation. JAK2, in addition to mediating GH signaling, stabilizes cell surface GHR by interacting with the membrane proximal region of the cytoplasmic domain GHR. This requires the N-terminus, not the kinase activity, of JAK2. However, whether JAK2 affects the ubiquitination, the internalization or other steps of intracellular trafficking of GHR is still unknown and needs to be further explored.

The binding of GH to GHR, apart from initiating GH signaling, also downregulates the receptor, thereby, restricting the magnititude and duration of the signaling. GH-induced GHR loss requires the presence of JAK2. Like the constitutive downregulation of GHR, GH-dependent GHR degradation also requires the Box1 element of GHR and N-terminus of JAK2, but not the JAK2 kinase domain. Whereas, JAK2 kinase activity is necessary for GH-induced GHR loss, and mutation of the tyrosine residues of the cytoplasmic domain of GHR to phenylalanine disrupts this effect of GH. However, whether the presence of the tyrosine residues per se or phosphorylation of these residues is required needs to be further examined. We also detected that either the deficiency of JAK2 kinase activity or mutation of the tyrosine residues of GHR blocked GH-induced GHR ubiquitination. Although the E3 ligase(s) involved in this process is still unclear, β-TrCP has been found to be involved in the degradation of GHR. Tyrosine mutation of GHR diminished GH-induced GHR internalization. However, whether or not JAK2 kinase deficiency affects GH-induced GHR internalization and whether the intracellular trafficking of GHR are impaired when JAK2 is deficient or GHR tyrosine residues are mutated will be further investigated.

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