Growth Hormone Receptor Ubiquitination, Endocytosis, and Degradation Are Independent of Signal Transduction via Janus Kinase 2*

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Cristina M. Alves dos Santos, Toine ten Broeke, and Ger J. Strous‡

From the Department of Cell Biology, University Medical Center Utrecht and Institute of Biomembranes, Heidelberglaan 100, AZU-G02.525, 3584 CX Utrecht, The Netherlands

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

GH regulates important physiological processes such as growth, metabolism, and cellular differentiation. The actions of GH are mediated through activation of the GHR, a member of the cytokine/hematopoietin receptor superfamily with homologies defined in the extracellular domain and that lacks intrinsic tyrosine kinase activity in its intracellular domain (1–3). Upon GH binding, the dimerized complex associates with the tyrosine kinase JAK2, a member of the Janus family of cytokine kinases (3–5). GH-dependent tyrosine phosphorylation of JAK2 itself, of GHR, and of other cellular proteins depends on the receptor’s ability to activate JAK2 (3–9). Biochemical evidence has shown that JAK2 activation by the GHR is essential for activating the signal transducer and activator of transcription proteins, several proteins involved in the Ras/MAP kinase pathway, and the insulin receptor substrate proteins IRS-1 and IRS-2, which initiate the phosphatidylinositol 3-kinase pathway (4, 9, 10, 11). Thus far, only the GH-dependent effect on calcium entry appears to involve mechanisms independent of JAK2 activation (12). Because a C-terminally truncated receptor able to interact with and activate JAK2 was unable to activate specific signaling molecules such as signal transducer and activator of transcription 5 (13), activation of JAK2 alone is insufficient to elicit all of the responses to GH, suggesting that such proteins are unlikely to be direct JAK2 substrates.

A proline-rich motif termed box-1 between amino acids 297 and 311 is conserved within members of the cytokine family and is required for the association of JAK2 with GHR as well as GH-dependent activation of JAK2 (1, 4, 14). Studies using mutated box-1 regions of the GHR have shown that this region is intermediate in GH-dependent association and activation of JAK2 (4, 7). Although box-1 is sufficient to bind and activate JAK2, maximal JAK2 activation requires downstream residues in the half-proximal part of the GHR cytoplasmic domain. This more distal region appears to stabilize the interaction between the receptor and JAK2. Within box-1, no specific residue seems, in itself, crucial for the association. Neither mutation of each individual proline residue nor simultaneous mutation of the first two prolines in box-1 impaired JAK2 association to the receptor. On the other hand, simultaneous mutation of the four prolines abolished the capacity of the receptor to interact with and activate JAK2 and other signaling proteins (4, 15, 16). Thus, the proline-rich region is critical for GH signal transduction.

After binding to its receptor, GH internalizes via clathrin-coated pits and is degraded in lysosomes (17, 18). This process is regulated by both the ubiquitin system and the proteasome (19–22). For other signaling receptors containing tyrosine kinase activity, such as the epidermal growth factor (EGF) (23) and G protein-coupled receptors (24, 25), it was shown that kinase activity is necessary for their maximal internalization rate. In the EGF receptor, mutation of its intrinsic tyrosine kinase activity abolished both signaling transduction and ligand-induced down-regulation/endocytosis (26, 27), whereas other receptors (e.g. transferrin) were internalized through coated pits without any known kinase requirement (23).

Until now, a role for JAK2 activity in endocytosis of the GHR has not been described. The role of box-1 in endocytosis is unclear, and mutations in box-1 may lead to a block of GHR endocytosis (15). Recently, we observed that signaling of GHR/JAK2 is also regulated by the proteasome because proteasomal inhibitor MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. When proteasomal inhibitor was...
combined with ligand in an endocytosis-deficient GHR mutant, the same phenomena occurred, indicating that proteosomal action on tyrosine dephosphorylation is independent of endocytosis (28). To determine the role of JAK2-mediated signal transduction in GHR internalization and down-regulation, we replaced the four proline residues in box-1 with alanines (GHR4P-A). Modification of box-1 resulted in the complete absence of GHR and JAK2 phosphorylation. We demonstrate that the GHR4P-A mutant behaves in a similar manner as the wtGHR. GHR4P-A presents the same internalization kinetics as wtGHR; it is ubiquitinated normally and depends on an intact ubiquitin system as well as on the proteasome for its internalization.

EXPERIMENTAL PROCEDURES

Cells and Materials—Chinese hamster ts20 cells were stably transfected with the full-length rabbit GHR cDNA sequence as described previously (19), as well as a cDNA encoding for a GHR mutation on box-1 (see "Mutagenesis and Transfection"). Due to a thermolabile ubiquitin-activating enzyme (E1), the ubiquitin conjugating system is inactive in these cells at the nonpermissive temperature of 42 °C. Stable Geniticin-resistant transfected were grown in Geneticin based on the 0.45 mg/ml. If necessary, MG132 (20 μM) dissolved in ethanol was added 1 h before the start of the experiment. Cells were washed free of unbound GH and incubated from 0 to 1 h at 30 °C. The cells were washed with ice-cold PBS, membrane-associated GH was removed by acid wash (0.15 M NaCl and 0.05 M glycine, pH 2.5), and internalized GH was determined by measuring the radioactivity after solubilization of the acid-treated cells by 1 N NaOH. The cell extracts were counted in a LKB gamma counter.

Microscopy—Cy3-GH was prepared using a FluoLink Cy3 label kit according to the supplier’s instructions (Amersham Pharmacia Biotech). Transfected ts20 cells, grown on coverslips, were incubated with 8 nM human GH at 30 °C for 30 min. After fixation, the cells were embedded in Mowiol, and confocal laser scanning microscopy was performed using a Leica TSC 4D system.
RESULTS

JAK2 Binding and Activation Is Absent in a Box-1-mutated GHR—To determine whether activation of the GHR and downstream signaling components are involved in GH-dependent endocytosis, the four proline residues between amino acids 280–287 in box-1 of the cytosolic tail of the GHR were mutated to alanines (GHR<sub>4P-A</sub>). An intact box-1 is required for the association of JAK2 with GHR and GH-dependent activation of JAK2 (4, 7). A ts20 cell line stably expressing GHR<sub>4P-A</sub> with approximately the same number of GH binding sites at the cell surface as the wild-type GHR ts20 cell line was used in this experiment. Immunoblot analysis of cellular extracts from both cell lines demonstrated that the mutant receptor expressed a GHR protein of the expected molecular size and that the level of total cell expression was comparable (Fig. 1A). To ascertain that the GHR<sub>4P-A</sub> mutant was unable to respond to GH, the tyrosine phosphorylation of the GHR was assayed. As shown in Fig. 1A, no PY signal was detectable on the 130-kDa GHR<sub>4P-A</sub> mutant upon GH stimulation, in contrast to wtGHR-expressing cells. As a negative control, untransfected ts20 cell lines were used. Similarly, JAK2 did not become tyrosine phosphorylated upon GH addition in cells expressing the GHR<sub>4P-A</sub> (Fig. 1B). Box-1 is therefore essential for activation of both JAK2 and GHR, confirming previously published results (4, 7). To test the capacity of the GHR<sub>4P-A</sub> to activate signaling molecules other than JAK2, a co-immunoprecipitation of GH-GHR complexes was performed and blotted for phosphotyrosine proteins (Fig. 2). As expected, the inactive GHR<sub>4P-A</sub> did not display the same phosphorylated bands as seen for the wtGHR, indicating that the mutant receptor did indeed lack signaling activity.

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

To approach the same question in a different way, a fibroblastoma cell line deficient in JAK2 (y2A) and its parental cell line containing JAK2 (2C4) transiently transfected with wtGHR were also tested for GH uptake. Both cell lines showed the same endocytosis rates of <sup>125</sup>I-GH as observed for the GHR<sub>4P-A</sub> mutant (data not shown).

Box-1 Is Not Involved in GHR Turnover—Next, we assessed the life cycle of both the wtGHR and the GHR<sub>4P-A</sub> mutant at 30°C through pulse-chase labeling with <sup>35</sup>S-methionine (Fig. 4). The wtGHR is synthesized as a 110-kDa glycoprotein precursor and converted thereafter to a 130-kDa mature species. For both the wtGHR and the GHR<sub>4P-A</sub>, the mature form was detectable at 20 min of chase and maximal at 60 min, after which rapid degradation occurred (Fig. 4, A and B). These results demonstrate that both receptors have a fast turnover.

Cell Lysis, Immunoprecipitation, and Western Blotting—Cells grown in dishes were lysed on ice in lysis buffer containing 1% Triton-100, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were cleared by centrifugation. In ubiquitin blotting experiments, the immunoprecipitates were washed twice with the same buffer and twice with PBS. For co-immunoprecipitations, cells were lysed with 10-fold diluted PBS. For immunoblotting experiments, the cells were lysed in boiling lysis buffer containing 1% SDS in PBS to minimize isopeptidase activity. The lysate was heated at 100°C for 5 min, and the lysates were further incubated with GH receptor antibody for 2 h, and the immunocomplexes were isolated through protein A-agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immunocomplexes. The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. For co-immunoprecipitations, cells were lysed in 0.1% Triton X-100, 0.5% BSA, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were further incubated with GH antibody for 2 h, and the immunocomplexes were isolated through protein A-agarose beads. All immunocomplexes were analyzed through 7.5% polyacrylamide gel electrophoresis in the presence of SDS (SDS-polyacrylamide gel electrophoresis) together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained with either Mab 4G10 (anti-PY), anti-ubiquitin, anti-GHR, or commercial JAK2 antibody. After incubating the blots with rabbit anti-mouse IgG (RAMPO) or protein A conjugated to horseradish peroxidase, antigens were visualized through the ECL system (Roche Molecular Biochemicals).

Metabolic Labeling—For metabolic labeling, the cells were incubated in methionine-free MEM for 30 min, and then <sup>35</sup>S-methionine (3.7 MBq/ml TRAN-<sup>35</sup>S label<sup>TM</sup>, 40 TBq/mmol; ICN, Costa Mesa, CA) was added and labeled for 10 min at 30°C in a CO<sub>2</sub> incubator. The radioactivity was chased for different times with or without 8 nM GH in the presence of MEM containing 0.1 mM unlabeled methionine and 0.1% BSA. The radioactivity was determined using a Storm<sup>TM</sup> imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image Quant software version 4.2a.
with a half-life of 45–60 min and that maturation and transport to the plasma membrane were not affected by a mutated box-1 in the GHR. To determine whether GH affects receptor turnover, the same experiment was performed in the presence of GH (Fig. 4, C and D). GH slightly accelerated degradation of the wtGHR mature form (Fig. 4C) as compared with GHR

Box-1 and Ubiquitin-Proteasome System-dependent Internal-
REAL TEXT

**DISCUSSION**

The GHR is a member of the cytokine/hematopoietin receptor superfamily, defined on the basis of a limited amino acid homology. In the intracellular domain of several members of this superfamily situated close to the plasma membrane, a proline-rich, 8-amino acid-long motif referred to as box-1 has been recognized as being involved in signal transduction (4, 7). The association of JAK2 to box-1 of the GHR, activation of the dimerized JAK2 kinase, and JAK2 autophosphorylation are all early steps in the signaling pathway (6). The central role of JAK2 in GH signaling is evident from studies using mutated GHRs that failed to bind or activate JAK2 and downstream effectors such as Src homology 2 containing protein (SHC), MAP, signal transducer and activator of transcription, and IRS (4, 9, 10, 11, 30). The results of our experiments shown in Fig. 1 confirm that JAK2 can be specifically tyrosine phosphorylated in response to GH. Mutation of the four prolines of the box-1 region at the membrane proximal domain of the cytoplasmic tail of the GHR abrogated JAK2 association with the GHR cytoplasmic domain as demonstrated previously (4). Although mutation of the box-1 region in the GHR did not affect the ability of the altered receptor to bind GH at the cell surface, it dramatically affected the receptor’s capacity to couple GH binding to JAK2 activation and consequently the capacity of other possible signaling molecules (Fig. 2). Interestingly, the biological responses of the GHR expressed in a variety of cell types (i.e., activation of MAP kinase, activation of c-fos gene expression, increased protein synthesis, lipid synthesis, and cellular proliferation) have not been observed for the GHR mutant of the box-1 region (30–32).

In contrast to what has been found with other families of signal-transducing growth factor receptors (e.g., EGF receptor), GH-stimulated JAK2 kinase activity and tyrosine phosphorylation of the GHR itself were not required for efficient GH-dependent internalization of the GHR (Fig. 3). A previous report suggested that mutations in box-1 might lead to a block of GHR endocytosis (15). In this study, the specific initial rate of internalization of the GHR4P-A appears to be nearly the same as that for normal receptor. Furthermore, as observed in Fig. 3, GH is still normally targeted for degradation in GHR4P-A, indicating that transport to the lysosomes is not affected in box-1-defective receptors. The GHR4P-A turnover also does not differ from that of the wtGHR, suggesting that, once internalized, intracellular sorting destinations of inactive receptor isoforms do not differ from those of functional receptor isoforms. However, upon GH stimulation, the wtGHR mature form showed relatively faster degradation, whereas the addition of GH had no such clear effect on the turnover of the GHR4P-A (Fig. 4). This result suggests that GH-dependent accelerated degradation of wtGHR is mediated through a JAK2-dependent process that is
absent in GHR_{4P-A}, indicating that signal transduction is contributing to a faster endocytosis/degradation of the GHR. This might be due to the fact that the signal transduction pathway via signal transducer and activator of transcription or MAP kinase stimulates clathrin-mediated endocytosis, a phenomenon known from EGF receptor studies (33).

Previously, we showed that the ubiquitin system is required for ligand-induced GHR internalization (19). In particular, a specific 10-amino acid sequence between amino acids 323 and 332 termed the UbE motif within the GHR cytosolic tail is involved in both GHR ubiquitination and ligand-induced endocytosis (21). GHR internalization requires the recruitment of the ubiquitin conjugation system to the UbE motif. Recently, we showed that the proteasome is also involved in GHR down-regulation (22). Specific proteasomal inhibitors block GH uptake of the full-length GHR, whereas a truncated receptor can endocytose undisturbed. Using ts20 cells, we determined whether the endocytosis of an inactive GHR is still inhibited upon inactivation of the ubiquitin system or upon inhibition of the proteasome with MG132. Our results show that at the presence or the activity of JAK2 and its signaling molecules.

In a recently published work, we showed that proteasomes are involved in the down-regulation of GHR activation signals (28). The endocytosis-deficient receptor GHR_{F327A} was still able to become phosphorylated and induce signal transduction. Surprisingly, its signal transduction was still down-regulated at the cell surface, indicating that it occurs independent of endocytosis. However, proteasomal inhibitors prevented GHR and JAK2 dephosphorylation at the cell surface. We concluded that the ubiquitin-proteasome system is a regulator of JAK2 signal transduction, probably via suppressors of cytokine signaling. Thus, the ubiquitin-proteasome system independently regulates the signal transduction capacity of the GHR in two ways: first, it determines the rate of endocytosis via the UbE-motif in the GHR tail, and second, it determines the signaling time via JAK2 and suppressors of cytokine signaling. Both mechanisms appear to be independently regulated by the same system, probably via completely different E2/E3 enzyme systems.

In other systems, e.g. the EGF receptor, association of the adapter protein Grb2 is required for receptor endocytosis. Grb2 associates with the cytoplasmic tail of the EGF receptor after stimulation by EGF, leading to activation of the Ras/MAP kinase signaling pathway. Grb2 also indirectly associates with the GHR via SHC in the region that maps to the distal cytoplasmic tail (amino acids 454–620) (35). Cells expressing truncated receptors, which lack this domain and are therefore unable to associate with Grb2F, can internalize the truncated receptor as efficiently as cells containing wtGHR. Thus, in addition to the dispensability of JAK2 activity and receptor tyrosine phosphorylation, recruitment of Grb2 to the receptor and activation of the MAP kinase pathway is also not required for GHR down-regulation. Similarly, EGF-stimulated activation of Src kinase leads to tyrosine phosphorylation of clathrin, and because phosphorylation is required to recruit clathrin to the membrane, this observation strongly suggests a role for c-Src in EGF receptor endocytosis (34). This is not the case for the GHR_{4P-A}, because inhibition of JAK2 and consequently of Src does not affect GHR clathrin-dependent endocytosis. Furthermore, it has recently been published that neither activation of the erythropoietin receptor, another member of the cytokine family, nor JAK2 tyrosine kinase activity is required for internalization of bound erythropoietin (36). Taken together, these results suggest that, for signaling receptors of the cytokine receptor superfamily, endocytosis follows a pathway distinct from signaling receptors of the receptor-tyrosine kinase family.

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