Chapter III

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

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GHR internalization and signal transduction are independently regulated by the ubiquitin system

Abstract

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

GH regulates important physiological processes such as growth, metabolism and cellular differentiation. The actions of GH are mediated through activation of the GHR, a member of the cytokine/hematopoietin receptor superfamily with homologies defined in the extracellular domain and that lacks intrinsic tyrosine kinase activity in its intracellular domain (1-3). Upon GH binding, the dimerized complex associates with the tyrosine kinase JAK2, a member of the Janus family of cytosolic kinases (3-5). GH-dependent tyrosine phosphorylation of JAK2 itself, GHR, and of other cellular proteins depends on the receptor's ability to activate JAK2 (3-9). Biochemical evidence has shown that JAK2 activation by the GHR is essential for activating the STAT proteins, several proteins involved in the Ras/MAP kinase pathway, as well as the insulin receptor substrate proteins IRS-1 and IRS-2, which initiate the PI-3 kinase pathway (4, 9, 10, 11). So far, only the GH-dependent effect on calcium entry appears to involve mechanisms independent of JAK2 activation (12). Since a C-terminally truncated receptor, able to interact with and to activate JAK2, was unable to activate specific signaling molecules, like STAT5 (13), activation of JAK2 alone is insufficient to elicit all of the responses to GH, suggesting that such proteins are unlikely to be direct JAK2 substrates.

A proline-rich motif termed box-1, between amino-acids 297-311, is conserved within members of the cytokine family and is required for the association of JAK2 with GHR as well as GH-dependent activation of JAK2 (1, 4, 14). Studies using mutated box-1 regions of the GHR have shown that this region is intermediate in GH-dependent association and activation of JAK2 (4, 7). Although box-1 is sufficient to bind and activate JAK2, a maximal JAK2 activation requires downstream residues in the half-proximal part of the GHR cytoplasmic domain. This more distal region appears to stabilize the interaction between the receptor and JAK2. Within box-1, no specific residue seems, in itself, crucial for the association. Mutation of each individual proline residue nor simultaneous mutation of the first two prolines in box-1 did impair JAK2 association to the receptor. On the other hand, simultaneous mutation of the 4 prolines abolished the capacity of the receptor to interact and to activate JAK2, as well as other signaling proteins (4, 15, 16). Thus, the proline-rich region is critical for GH signal transduction.

After binding to its receptor, GH internalizes via clathrin-coated pits and is degraded in lysosomes (17,18). This process is regulated by both the ubiquitin system and the proteasome (19-22). For other signaling receptors containing tyrosine kinase activity, like the epidermal growth factor (EGF) (23) and G protein-coupled receptors (24, 25), it was shown that kinase activity is necessary for their maximal internalization rate. In the EGF receptor, mutation of its intrinsic tyrosine kinase activity abolished both signaling transduction and ligand-induced downregulation/endocytosis (26, 27), while other receptors (e.g. transferrin) were internalized through
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coated pits without any known kinase requirement (23). Until now a role for JAK2 kinase activity of the GHR in endocytosis has not been described. The role of box-1 in endocytosis is unclear and mutations in box-1 may lead to a block of GHR endocytosis (15). Recently, we observed that signalling of GHR/JAK2 is also regulated by the proteasome, as proteasomal inhibitor MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. If proteasomal inhibitor was combined with ligand in an endocytosis-deficient GHR mutant, the same phenomena occurred indicating that proteasomal action on tyrosine dephosphorylation is independent of endocytosis (28). To determine the role of JAK2-mediated signal transduction in GHR internalization and downregulation, we replaced the four proline residues in box-1 with alanines (GHR_{P-A}). Modification of the box-1 resulted in the complete absence of GHR and JAK2 phosphorylation. We demonstrate that the GHR_{P-A} mutant behaves in a similar manner as the wtGHR. GHR_{P-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 non-permissive temperature of 42°C. Stabile geneticin-resistant transfectants were grown in Eagle’s minimal essential medium (MEMα) supplemented with 10% fetal bovine serum, penicillin and streptomycin, 3.5 g/liter glucose and 0.45 mg/ml geneticin. For experiments, cells were grown in the absence of geneticin to approximately 80% confluence. Anti-GHR antiserum was raised against amino acids 271-381 (anti-GHR(T)) of the cytoplasmic tail as previously described (19). Antibody recognizing the lumenal part of the GHR (Mab5) was from AGEn Inc., Parsippany, NJ. Antiserum against JAK2 was raised in rabbits as described in (29). Polyclonal antibody against JAK2 and phosphotyrosine (4G10, anti-PY) were obtained from Upstate Bio-technologies Inc. (Lake Placid, NY). GH antiserum was from DAKOPATTS and antiserum specific for protein-ubiquitin conjugates was a generous gift from Dr. A. Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). hGH was a gift of Lilly Research Labs, Indianapolis, IN. Culture medium, fetal calf serum (FCS) and geneticin were purchased from Gibco. MG132 was purchased from CalBiochem.
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Mutagenesis and transfection
The signaling-deficient mutant GHR4ΔA was constructed by QuickChange mutagenesis (Stratagene), using a 5′ oligonucleotide and a 3′ oligonucleotide containing the unique restriction site KpnI. This GHR cDNA was cloned into pcDNA3 vector (Invitrogen), sequenced and transfected into ts20 cells at 30% confluence using the calcium phosphate transfection method. After transfection, individual clones were selected in geneticin based on the amount of GH binding and by immunoblotting cell extracts with an antibody to the N-terminus of the GHR.

GH binding and internalization

125I-labeled hGH (125I-GH) was prepared by using chloramine T (19). Cells were grown in 35-mm dishes, washed and incubated with MEMα supplemented with 20 mM Hepes (MEMα/Hepes) and 0.1% bovine serum albumin (BSA) for 1 hour at 30°C, and further incubated for 1 hour on ice with 8 nM 125I-GH in MEMα/Hepes, in the absence or presence of excess unlabeled GH. The cells were washed free of unbound GH and incubated from 0 to 1 hours at 30°C. The cells were washed with ice-cold PBS, membrane-associated GH was removed by acid wash (0.15 M NaCl, 0.05 M glycine, pH 2.5), and internalized GH was determined by measuring the radioactivity after solubilization of the acid-treated cells by 1 M NaOH. The cell extracts were counted in a LKB gamma counter.

Microscopy

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

Cell lysis, immunoprecipitation and western blotting

Cells, grown in dishes, were lysed on ice in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were cleared by centrifugation. In ubiquitin blotting experiments, the cells were lysed in boiling lysis buffer containing 1% SDS in PBS in order to minimize isopeptidase activity. The lysate was heated at 100°C for 5 min, sheared to break all DNA, and centrifuged for 5 min at 10,000 x g. Immunoprecipitations were carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin
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(BSA), 1 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS. Reactions were incubated with GHR(T) or JAK2 antiserum for 2 hours at 0°C. Protein A-agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immuno-complexes. The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. For co-immunoprecipitations, cells were lysed in 0.1% Triton X-100, 0.5% BSA, 1 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride in PBS. The lysates were further incubated with GH antibody for 2 hours and the immuno-complexes were isolated through protein A-agarose beads. All immuno-complexes were analyzed through 7.5% polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained with either Mab 4G10 (anti-PY), anti-ubiquitin, anti-GHR, or commercial JAK2 antibody. After incubating the blots with rabbit anti-mouse IgG (RAMPO) or protein A conjugated to HRP, antigens were visualized through the ECL system (Boehringer Mannheim).

Metabolic labeling
For metabolic labeling, the cells were incubated in methionine-free MEM for 30 min and then [35S]methionine (3.7 MBq/ml TRAN-35S labelTM, 40 TBq/mmol, ICN, Costa Mesa, CA) was added and labeled for 10 min at 30°C in a CO₂-incubator. The radioactivity was chased for different times with or without 8 nM GH in the presence of MEMα containing 0.1 mM unlabeled methionine and 0.1% BSA. The radioactivity was determined using a StormTM imaging system (Molecular Dynamics, Sunnyvale, CA) and quantified with Molecular Dynamics Image QuaNT software version 4.2a.

Results

JAK2 binding and activation is absent in a box-1 mutated GHR
To determine whether activation of the GHR and downstream signaling components are involved in GH-dependent endocytosis, the 4 proline residues between amino-acids 280-287 in box-1 of the cytosolic tail of the GHR were mutated to alanines (GHR₄P₄A). 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 GHR₄P₄A 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 GHR₄P₄A 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_{4P-A} mutant upon GH stimulation, in contrast to wtGHR expressing cells. As a negative control, untransfected ts20 cell lines were used. Similarly, JAK2 did not become tyrosine phosphorylated upon GH in cells expressing the GHR_{4P-A} (Fig. 1B). Box-1 is, therefore, essential for activation of both JAK2 and GHR, confirming previously published results (4, 7). To test the capacity of the GHR_{4P-A} to activate signaling molecules other than JAK2, a co-immunoprecipitation of GH-GHR complexes was performed and blotted for phosphotyrosine proteins (Fig. 2). As expected, the inactive GHR_{4P-A} did not display the same phosphorylated bands as seen for the wtGHR, indicating that, indeed, the mutant receptor lacked signaling activity.

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

Fig. 2. **Tyrosine phosphorylation capacity of GHR_{4P-A} cells.**
Ts20 cells and cells expressing wtGHR and GHR_{4P-A} were incubated at 30°C with or without 8 nM hGH for the time periods indicated. Cells were lysed and GH-GHR complexes were co-immunoprecipitated with an antibody against GH (anti-GH), blotted with anti-PY (upper panel) and reblootted with anti-GHR (Mab5) (lower panel). Arrows indicate mature (mGHR) and precursor (pGHR) forms of the GHR.
JAK2 tyrosine kinase activity is not required for GH-dependent internalization of GHR.

To determine whether JAK2 tyrosine kinase activity is required for ligand-induced internalization, the uptake of 125I-GH was performed in cell lines expressing either wtGHR or the GHR4P-A mutant. As seen in Fig. 3, the percentage of internalization reached 50% after 30 min of GH induction for the wtGHR. In two independent cell lines, the rate of GH internalization for the box-1-mutated GHR was similar or higher than that of wild-type receptors. In order to exclude a possible contribution of receptor recycling, GH internalization was measured during the first 10 min of GH uptake and gave similar results for both types of receptors (not shown). In addition, the extent and rate of GH degradation were unaltered, indicating that JAK2 activation and GHR phosphorylation play no role in the transport of the ligand to the lysosomes. GHR4P-A internalization bound GH as efficiently as wtGHR, indicating that tyrosine phosphorylation of the GHR is not required for GH-dependent GHR internalization.

To approach the same question in a different way, a fibroblastoma cell line deficient in JAK2 (γ2A) and its parental cell line containing JAK2 (2C4) transiently transfected with wtGHR were also tested for GH-uptake. Both cell lines showed the same endocytosis rates of 125I-GH as observed for the GHR4P-A mutant (not shown).

Box-1 is not involved in GHR turnover

Next, we assessed the life-cycle of both the wtGHR and the GHR4P-A mutant at 30°C through pulse-chase labeling with [35S]methionine (Fig. 4). The wtGHR is synthesized as an 110 kDa glycoprotein precursor and thereafter converted to an 130 kDa mature species. For both the wtGHR and the GHR4P-A, the mature form was detectable at 20 min of chase and is maximal at 60 min, whereafter rapid degradation occurred (Fig. 4 A and B). These results demonstrate that both receptors have
a fast turnover with a half-life of 45-60 min and that maturation and transport to the plasma membrane were not affected by a mutated box-1 in the GHR. To determine whether GH affects receptor turnover, the same experiment was performed in the presence of GH (Fig. 4, C and D). GH slightly accelerated degradation of the wtGHR mature form (Fig. 4C), as compared to GHR4P-A (Fig. 4D).

**Box-1 and ubiquitin/proteasome system-dependent internalization**

Using ts20 cell lines, we showed previously that an active ubiquitin/proteasome system is essential for wtGHR internalization (19, 22). To elucidate this further, immunofluorescence studies were performed following the uptake of cy3-labeled GH for 30 min (Fig. 5). As a control, wtGHR cells were used (Fig. 5, A, B, C), clearly demonstrating an inhibition of internalization upon MG132 or 42°C pre-incubation, similar to previous results (19, 22). For the GHR4P-A mutant cells (Fig. 5, D, E, F), both treatments induced similar inhibitory effects as for the wtGHR. Fig. 5D confirms the results presented in Fig.3, clearly showing a normal ligand internalization staining pattern. Under conditions where either the proteasomes were inhibited (MG132) or the ubiquitin system was blocked (42°C), cy3-GH-dependent GHR4P-A mutant internalization was inhibited. To test whether kinase activity plays a role in GHR internalization, both cy3-GH and Texas Red-labeling...
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Tyrosine kinase activity is not essential for GHR endocytosis.

Ubiquitination of the GHR is independent of its phosphorylation and signal transduction

To determine whether GHR signaling is involved in receptor ubiquitination, we tested whether the GHR4P-A mutant was ubiquitinated upon GH stimulation. As shown in Fig.6, in the presence of GH, wtGHR was ubiquitinated at the permissive temperature but not ubiquitinated at the non-permissive temperature of 42°C. Similar results were obtained for the GHR4P-A. Thus, the ubiquitin-proteasome system does not require JAK2 action nor its presence on the dimerized GHR for its action to stimulate receptor endocytosis. In conclusion, GHR signal transduction via JAK2 is independent of ubiquitination, endocytosis and degradation.

Discussion

The GHR is a member of the cytokine/hematopoietin receptor superfamily, defined on the basis of a limited amino acid homology. In the intracellular domain of several members of this superfamily situated close to the plasma membrane, a proline-rich motif eight amino acids residues long, referred to as box-1, has been recognized as being involved in signal transduction (4, 7). The association of JAK2 to box-1 of the GHR, activation of the dimerized JAK2 kinase, and JAK2 autophosphorylation are all early steps in the signaling pathway (6). The central role of JAK2 in GH signaling is evident from studies using mutated GHRs which failed to bind or activate JAK2 and downstream effectors like SHC (Src homology (SH)-2 con-
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...taining protein), MAP, STAT, and IRS (4, 9, 10, 11, 30).

The results of our experiments shown in Fig. 1, confirm that JAK2 can be specifically tyrosine phosphorylated in response to GH. Mutation of the four prolines of box-1 region at the membrane proximal domain of the cytoplasmic tail of the GHR abrogated JAK2 association with the GHR cytoplasmic domain as previously demonstrated (4). Although mutation of the box-1 region in the GHR did not affect the ability of the altered receptor to bind GH at the cell-surface, it dramatically affected the receptor's capacity to couple GH binding to JAK2 activation and consequently of other possible signaling molecules (Fig. 2). Interestingly, the biological responses of the GHR expressed in a variety of cell types (i.e. activation of MAP kinase, activation of c-fos gene expression, increased protein synthesis, lipid synthesis, and cellular proliferation), have not been observed for the GHR mutant of the box-1 region (30, 31, 32).

In contrast to what has been found with other families of signal-transducing growth factor receptors (e.g., EGF receptor), GH-stimulated JAK2 kinase activity and tyrosine phosphorylation of the GHR itself were not required for efficient GH-dependent internalization of the GHR (Fig. 3). A previous report suggested that mutations in box-1 may lead to a block of GHR endocytosis (15). In this study, the specific initial rate of internalization of the GHR\textsubscript{4P-A} appears to be nearly the same as for normal receptor. Furthermore, as observed in Fig. 3, GH is still normally targeted for degradation in GHR\textsubscript{4P-A}, indicating that transport to the lysosome is not affected in box-1-defective receptors. The GHR\textsubscript{4P-A} turnover also does not differ from the wtGHR, suggesting that, once-internalized, intracellular sorting destinations of inactive receptor isoforms do not differ from those of functional receptor isoforms. However, upon GH stimulation, wtGHR mature form showed relatively faster degradation, while addition of GH had no such clear effect on the turnover of the...

Fig. 6. Effect of box-1 mutation in the GHR on ubiquitination. Cells expressing wtGHR and GHR\textsubscript{4P-A} were incubated for 1 hour at 30 or 42°C and treated with 8 nM GH for 30 min. After lysis, GH was immunoprecipitated with anti-GHR (T) and analyzed by Western blotting using an anti-ubiquitin conjugate serum (anti-Ubi). As a control, untransfected cells (ts20) were subjected to the same procedure. A reblot with anti-GHR (Mab5) is shown on the lower panel.
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GHR<sub>4P,A</sub> (Fig. 4). This result suggests that GH-dependent wtGHR accelerated degradation is mediated through a JAK2-dependent process that is absent in GHR<sub>4P,A</sub>, indicating that signal transduction is contributing to a faster endocytosis/degradation of the GHR. This might be due to the fact that the signal transduction pathway via STAT or MAP kinase stimulates the clathrin-mediated endocytosis, a phenomenon known from the EGF receptor studies (33).

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

In a recently published work, we showed that proteasomes are involved in the downregulation of the GHR activation signals (28). The endocytosis-deficient receptor GHR F327A, was still able to become phosphorylated and to induce signal transduction. Surprisingly, its signal transduction was still downregulated at the cell-surface, indicating that it occurs independent of endocytosis. However, proteasomal inhibitors prevented GHR and JAK2 dephosphorylation at the cell-surface. We concluded that the ubiquitin-proteasome system is a regulator of JAK2 signal transduction, probably via suppressors of cytokine signalling (SOCS). Thus, the ubiquitin-proteasome system independently regulates the signal transduction capacity of the GHR in two ways: first, it determines the rate of endocytosis via the UbE-motif in the GHR tail, and second, it determines the signaling time via JAK2 and SOCS. Both mechanisms appear to be independently regulated by the same sys-
tem, probably via completely different E2/E3 enzyme systems.
In other systems, e.g. the EGF receptor, association of the adapter protein Grb2 is required for receptor endocytosis. Grb2 associates with the cytoplasmic tail of the EGF receptor after stimulation by EGF, leading to activation of the Ras/MAPK signalling pathway. Grb2 also indirectly associates with the GHR via SHC in the region that maps to the distal cytoplasmic tail (amino acids 454-620) (35). Cells expressing truncated receptors that lack this domain and therefore unable to associate with Grb2, can internalize the truncated receptor as efficiently as cells containing wtGHR. Thus, in addition to the dispensability of JAK2 activity and receptor tyrosine phosphorylation, recruitment of Grb2 to the receptor and activation of the MAPK pathway is also not required for GHR downregulation. Similarly, EGF-stimulated activation of Src kinase leads to tyrosine phosphorylation of clathrin, and as phosphorylation is required to recruit clathrin to the membrane, this observation strongly suggests a role for c-Src in EGF receptor endocytosis (34). This is not the case for the GHR_{4P,4A}, since inhibition of JAK2 and consequently of Src does not affect GHR clathrin-dependent endocytosis. Furthermore, it has recently been published that neither activation of the erythropoietin receptor, another member of the cytokine family, nor JAK2 tyrosine kinase activity are required for internalization of bound erythropoietin (36). Taken together, these results suggest that, for signaling receptors of the cytokine receptor superfamily, endocytosis follows a pathway distinct from signaling receptors of the RTK family.

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