Chapter II

The Signal Transduction of the Growth Hormone Receptor Is Regulated by the Ubiquitin/Proteasome System and Continues After Endocytosis

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Journal of Biological Chemistry 276, 10839-10846, 2001
GHR signal transduction is regulated by the ubiquitin system and continues after endocytosis

Abstract

The growth hormone receptor (GHR) intracellular domain contains all of the information required for signal transduction as well as for endocytosis. Previously, we showed that the proteasome mediates the clathrin-mediated endocytosis of the GHR. Here we present evidence that the 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. Experiments with a GHR truncated tail mutant (GHR1-369) led to a prolonged JAK2 phosphorylation due to the loss of a phosphatase-binding site. This raised the question of what happens to the signal transduction of the GHR after its internalization. Co-immunoprecipitation of GH-GHR complexes before and after endocytosis showed that JAK2 as well as other activated proteins are bound to the GHR not only at the cell-surface but also intracellularly, suggesting that the GHR signal transduction continues in endosomes. Additionally, these results provide evidence that GHR is present in endosomes both in its full-length and truncated form, indicating that the receptor is downregulated by the proteasome.
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Introduction

The growth hormone receptor (GHR) is a member of the cytokine/hematopoietin receptor superfamily (for review see 1). Cytokines regulate different aspects of cellular growth, differentiation and activation and play a critical role in immune and inflammatory responses. In response to GH, two GHR polypeptides dimerize, turning on a cascade of events leading to signal transduction by activating gene transcription in the cell nucleus and, at the same time, downregulation and degradation of the receptor (2, 3).

One major characteristic of the cytokine receptor family is the absence of an intrinsic tyrosine kinase activity. Upon dimerization, the GHR recruits and activates JAK2, a member of the Janus family of cytosolic kinases (4-6). Once bound, the two JAK2 molecules are in opposition and can transphosphorylate each other. Subsequently, the receptor chains become tyrosine phosphorylated allowing them to interact with other intracellular signaling components (6). JAK2 acts via special signal transducers and activators of transcription proteins (STATs), which dimerize and translocate to the nucleus to convey the appropriate signal to specific regulatory DNA-response elements (7, 8). In addition, JAK2 activation by GH facilitates initiation of various pathways including the Ras, mitogen-activated protein kinase (MAPK), the insulin receptor substrate (IRS) and the phosphatidylinositol 3-kinase (PI-3K) pathway (9, 10). GH-induced activation of the JAK/STAT signal transduction pathway is both rapid and transient. The molecular mechanism of JAK deactivation is still poorly understood. Part of the dephosphorylation of the GHR has been previously attributed to the activation of the tyrosine phosphatase SHP-1 (11). This enzyme was found to interact with JAK2 and GH stimulates the catalytic activity of SHP-1 (11). Another candidate could be SHP-2 since it associates with the GHR, and binding to JAK2 has also been reported. However, no dephosphorylation of JAK2 by SHP-2 could be demonstrated (12). Recently, another negative regulatory pathway of the GH receptor signaling involving the SOCS (suppressor of cytokine signaling) proteins has been identified (13, 14). The SOCS proteins appear to form part of a negative feedback loop that regulates cytokine signal transduction. Their expression is rapidly induced by activation of the JAK/STAT pathway (15).

Another important system which downregulates the GHR is the ubiquitin-proteasome system. This system regulates the degradation of nuclear and cytosolic proteins via the proteasome (16). The target proteins are first tagged with ubiquitin molecules to form a polyubiquitin chain, which is specifically recognized by the multi-subunit proteasome complex, leading to their degradation. Proteasomes were found to be involved in regulating JAK/STAT pathways upon interleukin-2, -3 and erythropoietin stimulation (17-19). In the presence of specific proteasomal inhibitors, activation of both JAK and STAT molecules was sustained, although neither STAT nor JAK appeared to be ubiquitinated. These data indicate that protea-
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Some are involved in the downregulation of the activation signals of specific cytokine receptors. An important factor in GHR down-regulation is its endocytosis. In the presence of ligand GHR endocytoses rapidly via clathrin-coated pits (20), and its degradation occurs at least partially within the lysosome (21). The ubiquitin system is required for ligand-induced GHR internalization (14). In particular, the UbE motif within the GHR cytosolic tail is involved in both GHR ubiquitination and ligand-induced endocytosis (22). In a chinese hamster cell line carrying a temperature-sensitive E1 enzyme (ts20 cells), inactivation of E1 results in accumulation of non-ubiquitinated GHRs at the plasma membrane, while internalization of the transferrin receptor is unaffected (23). Recently, we showed that the proteasome is also involved in GHR downregulation (24). GHR internalization requires proteosomal action in addition to an active ubiquitin conjugation system. Specific proteosomal inhibitors block GH uptake of the full-length GHR, while a truncated receptor can endocytose undisturbed.

In this report, we address the role of proteasome-mediated protein degradation in modulating GHR/JAK2 activity following GH stimulation. We show that the proteosomal inhibitor MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. Furthermore, we observe that JAK2 is not only bound to the GHR at the cell-surface but also intracellularly, suggesting that the receptor and other signal transducing molecules are still active in endosomes.

Experimental procedures

Cells and Materials
Chinese hamster ts20 cells were stably transfected as described previously (23). Truncated receptors were constructed by introducing stop codons at various positions within the rabbit cDNA (25). These truncated GHR cDNAs were cloned in pcDNA3 (In Vitrogene Inc.) and transfected into ts20 cells, resulting in cell lines stably expressing receptors truncated at amino-acid residues 399 and 369. The internalization-deficient mutant GHR F327A was constructed by site-directed mutagenesis, cloned and transfected into ts20 cells as described before (25). Stable, geneticin-resistant transfectants were grown in Eagle's minimal essential medium (MEM-α) supplemented with 10% fetal bovine serum, penicillin and streptomycin, 4.5 g/liter glucose, and 0.45 mg/ml geneticin. For experiments, cells were grown in the absence of geneticin at approximately 70% confluence.

Antibody to GHR was raised against amino acids 271-381 of the cytoplasmic tail (anti-GHR(T)) as previously described (23). Antibody (Mab5) recognizing the lumenal part of the GHR was from AGEN Inc., Parsippany, NJ. Antiserum against JAK2 was raised in rabbits against a synthetic peptide corresponding to the hinge
region (amino acids 758-777) between domains 1 and 2 of murine JAK2. Polyclonal antibody against JAK2 and phosphotyrosine (4G10, anti-PY) were obtained from Upstate Bio-technologies Inc. (Lake Placid, NY). Antiserum against human GH was raised in rabbits. Commercial anti-GH was from DAKOPATTS. hGH was a gift of Lilly Research Labs, Indianapolis, IN. Culture medium, fetal calf serum (FCS) and geneticin were purchased from Gibco. MG132 was from CalBiochem.

**Cell lysis, immunoprecipitation and immunoblotting**

Cells, grown in 10-cm dishes, were first incubated for 2 hours at 30°C in FCS-free MEM-α- in presence or absence of 20 mM MG132. After hGH (8 nM) incubation, cells were lysed on ice in 0.6 ml of lysis mix containing 1% Triton X-100, 1 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4 and 50 mM NaF in PBS. The immunoprecipitations were performed in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin, 1 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4 and 50 mM NaF in PBS. The lysates were cleared by centrifugation and incubated with GHR antiserum or JAK2 antiserum for 2 hours on ice. Protein A-agarose beads (Repligen Co., Cambridge, MA) were used to isolate the immune complexes. The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. Immune complexes were analyzed by poly-acrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) together with total cellular lysate and transferred to polyvinylidene difluoride paper. The blots were immunostained using either Mab 4G10 (anti-PY), 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 using the ECL system (Boehringer Mannheim).

**Co-immunoprecipitation of internalized proteins**

Cells, grown on 10-cm dishes, were incubated 1 hour on ice in MEM-α, supplemented with 20 mM Heps, and with 8 nM hGH. The cells were then washed once to remove unbound GH and incubated at 30°C for different periods of time. Cells were put on ice after which the cell-surface labeled GH was removed by two times 30 sec with acidic solution of 50 mM glycine, 150 mM NaCl pH 2.5. The cells were washed with PBS and lysed in 0.1% Triton X-100, 1 mM EDTA, 0.5% BSA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4 and 50 mM NaF in PBS. Immunoprecipitations were performed in the same buffer with GH antiserum for 2 hours on ice. The immune complexes were treated as above. The blots were immunostained using Mab 4G10 or JAK2 antibody. For the acid wash control, a competition assay was performed by using unstimula-
Effect of specific proteasomal inhibitors on GHR phosphorylation

To determine whether the proteasome is involved in modulating GHR activity, we examined the effects of the proteasomal inhibitor MG132 on GH-induced GHR phosphorylation. Using ts20 cells stably transfected with wtGHR, the time course of tyrosine phosphorylation of the GHR was determined following GH stimulation in the presence and absence of MG132 (Fig. 1). In the absence of GH, no phosphorylated GHR was visible (Fig. 1A). Upon GH stimulation a broad 130-kDa band appeared, indicating the GHR tyrosine phosphorylation. The activity was maximal within 15 min and decreased thereafter. If MG132 was present, virtually no decrease of the GHR phosphorylation signal was observed even after two hours. Reblotting with an anti-GHR antibody showed equivalent amounts of total immunoreactive protein in all samples, indicating that MG132 had little effect on the steady-state level of the GHR (Fig. 1B). Since GH stimulation was continuous, it was not possible to discriminate the population of down-regulated receptors from newly synthesized receptors as done previously by a metabolically labeled pulse-chase experiment (24). Thus, prolonged phosphorylation of the receptor due to the presence of MG132 could account for the sustained presence of the receptor at the cell-surface.

Fig. 1. Effect of MG132 on GHR phosphorylation. Cells were incubated for 2 hours at 30°C without (control) or with 20 mM MG132 and supplemented with 8 nM hGH for the time periods indicated. Cell lysates were prepared and subjected to immunoprecipitation (IP) of the GHR by using anti-GHR(T) and subjected to immunoblot analysis with an antibody against phosphotyrosine residues (anti-PY). A, ts20 cells expressing the wtGHR. C, ts20 cells expressing the GHR F327A. B and D, the PY blots were reblotted with anti-GHR(T). Arrows indicate mature (mGHR) and precursor (pGHR) forms of the GHR.
The MG132 effect is not due to inhibition of internalization of GHR

Our previous results have shown that MG132 prevents internalization of the GHR (24). It is anticipated that a prolonged stay at the cell-surface might result in a prolonged phosphorylated state of the GHR and of JAK2. To test this, we used the GHR F327A transfected cells, which express receptors defective in internalization (25, 26). The kinetics of tyrosine phosphorylation/dephosphorylation of the GHR F327A were similar to the wtGHR (Fig. 1C), reaching a maximum after 15 min and decreasing to basal levels after 2 hours of GH treatment. However, in the presence of MG132, the level of tyrosine phosphorylation of the GHR F327A remained the same. Thus, down-regulation of the GHR phosphorylation depends on proteasomal action, and is not related to the GH-induced endocytosis.

GHR sustained activation is due to prolonged JAK2 phosphorylation

Proteasomal inhibitors prolong signaling of the interferon-gamma receptors after ligand stimulation, showing sustained tyrosine phosphorylation of both the receptors and JAK1/JAK3 (17). To determine whether the effect of MG132 on GHR phosphorylation is due to sustained activation of JAK2 kinase, anti-JAK2 immunoprecipitates were prepared from cell lysates and analyzed by immunoblotting with an antibody to phosphotyrosine. As shown in Fig. 2A, GH induced a transient phosphorylation of JAK2 with a maximum at 15 min in the absence of proteasomal inhibitor, declining to nearly basal levels after 2 hours. However, treatment of the cells with MG132 prevented the dephosphorylation of JAK2, correlating well with the sustained GHR activity (compare lanes 4 and 8). Reblotting with an anti-JAK2 antibody showed similar amounts of immunoreactive protein in all samples indicating that MG132 had little effect on the stability of the protein (Fig. 2B). Shorter
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Preincubation periods with MG132 were as effective in stabilizing the tyrosine phosphorylation of JAK2, suggesting that its mechanism of action is specific and not due to general cell toxic effects. The same was observed for the GHR F327A mutant (Fig. 2C and 2D). JAK2 activation was transient in this mutant, but as for the wtGHR, MG132 treatment prolonged JAK2 phosphorylation in a similar way. Taken together, these results demonstrate that MG132 prolongs the GH-induced activity of both GHR and JAK2, presumably through stabilization of GHR and JAK2 tyrosine phosphorylation. Thus, the proteasome plays a role in decreasing GHR signal transduction.

Possible role of proteasomes in modulating phosphatase activity

Previous reports have implicated JAK proteins in dephosphorylation by interaction with specific phosphatases (12,27-29). Hackett et al., using FDP-C1 cells, demonstrated that the region in the GHR cytosolic tail between 521 and 540 is required for inactivation of the JAK/STAT signaling cascade, possibly via the protein tyrosine phosphatase SHP-1 that acts as a negative regulator (11). However, SHP-1 does not seem to associate with the GHR. Also the tyrosine phosphatase SHP-2, another member of the protein-tyrosine phosphatase family that, unlike SHP-1, is ubiquitously expressed in vertebrate cells, was shown to form a complex with both the tyrosine phosphorylated receptor (GHR cytoplasmic domain residues 485-620) and JAK2 protein (30). We determined whether MG132 would also induce prolonged JAK2 phosphorylation upon GH treatment in a C-terminally truncated GHR. JAK2 was immunoprecipitated from GHR 1-399 and GHR 1-369 expressing cells and immunoblotted with antiphosphotyrosine antibodies for various times of GH treatment (Fig. 3A and C). In both cell lines, JAK2 showed tyrosine phosphorylation with no change during time. In accordance with the above-mentioned studies,
delayed dephosphorylation of the kinase was observed both in the presence and absence of MG132. Reprobing the blots with JAK2 antibody confirmed the presence of equal amounts of JAK2 protein in each sample (Fig. 3B and 3D). These results suggest that the activation of a negative regulator (SHP-1 or SHP-2) through distal GHR tail domains and further association with JAK2 might be the important factor responsible for down-regulating the GHR/JAK2 phosphorylation in a proteasome-dependent way. Thus, inhibition of the proteasome by MG132 inhibits the dephosphorylation of JAK2, resulting in prolonged activity of both JAK2 and GHR. However, MG132 had no effect on SHP-2 phosphorylation upon GH induction, or had any effect on SHP-2 binding to both GHR and JAK2 (results not shown).

As shown previously, proteasomal inhibitors do not affect internalization of GH via the GHR 1-369 but effectively block endocytosis of GHR 1-399 (24). As JAK2 phosphorylation is similar in both cell lines, the data implicate that signaling might continue after endocytosis.

### JAK2 protein is bound to GHR in endosomes

Signaling via the GHR begins at the cell-surface. As demonstrated above using the endocytosis-defective GHR F327A cell line, the activation/deactivation (tyrosine phosphorylation/ dephosphorylation) cycle can be initiated and completed at the

![Fig. 4. Acid-resistant GH-GHR complexes are found in the cell.](image-url)
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The next question is whether signal transduction can continue after endocytosis. To address this, the activity of GHR has to be established after endocytosis. To accomplish this, we isolated GH-GHR complexes using anti-GH immunoprecipitation after acid treatment (Fig. 4). Dissociation of GH-GHR complexes does not occur at (endosomal) pH 5.5 (20). This indicates that GH remains complexed to its receptor, independent of its intracellular routing, unless it is localized to the lysosome. In that case, the ligand as well as the receptor is rapidly degraded (21). Treating living cells with buffers of pH higher than 2.5 showed that GH was not removed from GHR at the cell-surface, and only upon treatment with a buffer pH 2.5 did GH detach from the receptor, without interfering with the already internalized GH-GHR (results not shown). If no acidic treatment was performed, the total amount of wtGHR bound to GH co-immunoprecipitated during the different periods of time (Fig. 4A, lanes 1-4). The same was observed for the truncated GHR 1-369 (Fig. 4B, lanes 9-12) and GHR F327A (Fig. 4D, lanes 26-29). If the cells were kept on ice, acid treatment removed virtually all the GH from the cell-surface and hardly any GHR was detectable (Fig. 4, lanes 5, 13, 21 and 30). Upon incubation at 30°C, GH became acid-resistant indicating that GH-GHR complexes had entered the cells. Within 15 min, both wtGHR and GHR 1-369 were detectable inside the cells. Longer periods of GH treatment resulted in a decrease of GH-bound

Fig. 5. Acid-treatment control. A, cells expressing wtGHR were treated with GH for 15 min and acid washed as previously, but lysis was performed in presence of cell extracts from unstimulated GHR 1-369 cells at 1, 2, or 3-fold excess concentrations of wtGHRs. The cell lysates were immunoprecipitated with anti-GH and blotted with anti-GHR (Mab5). B, total lysates were blotted with anti-GHR (Mab5).
internalized receptors in the endosomes (lanes 8 and 16). The GHR F327A was not observed inside the cells since internalization of this receptor is inhibited (lanes 30-33). In addition to the 130 kDa band of the GHR, a smear of bands (60-80 kDa) appeared which only reacted with Mab5 (Fig. 4A) and anti-GHR(T) (not shown) and not with anti-GHR C-terminal tail antibody (Fig. 4C). No degradation products were visible if GHR 1-369 was analyzed (not shown). These observations show that the partial degradation of the wtGHR starts from the C-terminus very soon after GH binding.

To exclude the possibility that during or after lysis GH is free to rebind endocytosed or cell-surface receptor, the same experiment for wtGHR was performed but excess of unstimulated GHR 1-369 lysate was added during lysis (Fig. 5). If free GH is available to react with wtGHR at the cell-surface or in endosomes, then an excess of GHR 1-369 will compete for binding to free GH. If this would be the case, then GH immunoprecipitates blotted with anti-GHR (Mab5) should present GH complexes with both wtGHR and GHR 1-369. As these receptors have different sizes but the same GH binding affinity, they can easily be distinguished by immunoblot. As observed on Fig. 5A, wtGHR cells were treated with GH for 15 min and acid treated. Addition of non-stimulated GHR 1-369 lysate in different concentrations, did not result in GH complexes containing the truncated receptor. Addition of excess of lysate of untransfected ts20 cells to the wtGHR expressing cells was also tested, with the same result. Fig. 5B shows total cell lysates blotted with anti-GHR (Mab5), indicating the amount of the truncated GHR 1-369 in the incubations. Performing the same experiment by lysing GH-treated GHR 1-369 cells in the presence of unstimulated wtGHR lysate, no GH-wtGHR complexes were detected (not shown).

We then addressed the question whether internalized GHR is able to bind JAK2. The same time course experiment was performed as in Fig. 4 and analyzed for JAK2 molecules (Fig. 6A). To measure the total amount of JAK2 bound to the GH-GHR complex, no acidification was performed (lanes 1-3). In wtGHR transfected cells upon acid wash, JAK2 co-immunoprecipitated with GH-GHR complexes after internalization (lanes 5 and 6). The same was observed in the GHR 1-369 mutant (lanes 7-9). A faster migrating background band reacted with anti-JAK2 after cell acidification, presumably due to proteolysis. As expected, the GHR F327A mutant did not show JAK2 binding after acidification. JAK2 was neither detectable in the anti-GH immunoprecipitates from untransfected ts20 cells (lane 13) nor unstimulated cells expressing the wtGHR, the GHR 1-369, and the GHR F327A (lanes 1, 4, 7 and 10), indicating the efficiency of the acid wash procedure. Similar amounts of JAK2 were found for the different cell lines as seen in Fig. 6B. These results show that JAK2 is bound to the GHR inside the cell, suggesting that the receptor is capable of signaling in endosomes.
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GHR signaling continues inside the cell
To determine if other proteins attached to the GH-GHR in endosomes are phosphorylated, cells expressing wtGHR and GHR 1-369 were treated as described above and analyzed for phosphotyrosine positive proteins. As seen in Fig. 7A, GH induced the phosphorylation of a set of high molecular weight proteins. Upon GH removal from the cell-surface, the same set of proteins in the higher molecular weight range, attached to the internalized GH-GHR complex, were phosphorylated. Untransfected ts20 cells only resulted in a background pattern. In this molecular weight range both wtGHR and JAK2 proteins are possible candidates, consistent with the results presented above. Surprisingly, both wild type and truncated GHR 1-369 presented similar pattern of phosphorylated proteins. This can be explained by the fact that signaling proteins mainly interact with the membrane proximal region of the cytosolic tail of the GHR via JAK2. Although mainly high molecular weight proteins were phosphorylated, we cannot exclude that also lower molecular weight proteins were activated since the immunoprecipitation might be ineffective or the amount of molecules insufficient to allow their detection. Similar amounts of the receptor were found in each lane as observed in Fig. 7B.

Discussion
The ubiquitin-proteasome system plays an essential role in many cellular regulatory processes including cell cycle progression, DNA repair, transcriptional control and cell-surface-associated receptor endocytosis. In all these processes the ubiquitin-conjugating system targets ubiquitinated proteins to the proteasome for degra-
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For the GHR, the ubiquitin system was found to be involved in GH-dependent endocytosis (31, for review see 32). In the present study, we demonstrate that the ubiquitin-proteasome system is involved in the down-regulation of GHR signal transducing events. Others have demonstrated that in several cytokine receptors the JAK/STAT pathway was downregulated by the proteasome. Both interleukin-2 and -3 and interferon receptor showed a prolonged JAK/STAT activation as well as other signaling molecules like MAP kinases, upon treatment with specific proteasomal inhibitors (18, 17). The studies described in this paper demonstrate a similar effect for the GHR. Using ts20 cells stably transfected with wtGHR, we show that, in the presence of the specific proteasomal inhibitor MG132, the phosphorylation/activation of both receptor and tyrosine kinase JAK2 are prolonged for long periods of GH induction. Furthermore, our data indicate that proteasomal action on signal transduction occurs at the cell-surface since signaling by the GHR F327A endocytosis-deficient mutant still depends on the proteasome for its down-regulation. These results support the notion that GHR/JAK2 signal down-regulation is not determined by endocytosis per se.

Which mechanisms underlie the down-regulation of the GHR and JAK2 proteins? Several reports have shown that tyrosine phosphatases are involved in the dephosphorylation of JAK proteins. Ligand-induced tyrosine phosphorylation/activation of JAK2 by erythropoietin receptor, induces binding of the protein tyrosine phos-

![Fig. 7. Phosphorylation of proteins attached via the GH-GHR complex after internalization.](image)

The same procedure as in Fig 4. Immunoprecipitates of GH-GHR complexes from wtGHR, GHR 1-369 and untransfected ts20 cells were blotted with anti-PY, and control blots of direct lysates were immunoblotted with anti-GHR(T), as shown respectively in A and B.
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phosphatase SHP-1 to the cytoplasmic domain of the receptor. The recruitment of SHP-1 is accompanied by dephosphorylation of JAK2 and subsequent termination of erythropoietin-induced cellular proliferation (33, 34). A similar role for SHP-1 in mediating the down-regulation of JAK2 following stimulation of cells with GH has been proposed (11). Our results with GHR 1-399 and GHR 1-369 indicate that partial deletion of the C-terminal GHR tail leads to a prolonged JAK2 phosphorylation presumably due to loss of a negative regulator binding site and its consequent activation. This pattern of prolonged phosphorylation is similar to that of JAK2 in full length wtGHGRs treated with MG132. One explanation might be that the phosphatase activity is modulated by proteasome function, perhaps by degrading an inhibiting complex in a similar manner as it occurs for the inhibitor of the transcription factor NF-κB (35). This would explain why in the presence of MG132, phosphatase inhibitors prevent dephosphorylation of the JAK2 by SHP-1, and thereby prolonging phosphorylation of both JAK2 and the GHR. In support of this model, SHP-1 degradation has been shown to be ubiquitin-dependent in mast cells (36), suggesting that the proteasome is involved in SHP-1 regulation. SHP-2, however, has been shown to interact directly with the tail of the GHR (residues 484-620) and associate with JAK2 and SIRPα1, a member of a family of transmembrane glycoproteins identified by its association to SH2 domain-containing SHP-2. In response to GH, JAK2 associates with SIRPα1 and rapidly stimulates tyrosine phosphorylation of both SIRPα1 and SHP-2, and enhances association of these two molecules (37). Recently, it was shown that SIRPα1 is acting as a negative regulator of GH signaling by its ability to bind SHP-2 (38). The proteasome could therefore play a role in SHP-2/SIRPα1 association and binding to JAK2. As SHP-2 is known to associate to other signaling molecules as IRS-1 (39) and p85-PI 3-K (40), future studies will indicate whether the MG132 effect on these molecules is directly related to SHP-2 activity. However, it cannot be excluded that activated JAK kinases themselves are subject to proteasome-mediated degradation. Support for this comes from a recent identified negative regulatory pathway of the GHR signaling involving the SOCS proteins. GH preferentially induces the rapid, transient expression of SOCS-3, a member of the SOCS family that is known to inhibit cytokine receptor signaling. Expression of other SOCS genes, SOCS-1, SOCS-2 and CIS, was also up-regulated by GH, although to a lesser extent than SOCS-3 and with different kinetics (14). Recently, it was shown that the highly conserved C-terminal homology domain of the SOCS proteins, termed the SOCS box, mediates interactions with elongins B and C, which in turn may couple SOCS proteins and their substrates to the proteasomal protein degradation pathway (41). How SOCS proteins inhibit JAK kinase activity is still not clear, but analogous to the family of F-box-containing proteins, SOCS box interaction with elongins B and C potentiates interaction with the proteasome complex. This would explain why, in presence of MG132, degradation of SOCS proteins and its associated proteins like JAK2 would
be prevented, and therefore induce sustained activation of JAK2 and, consequently the GHR. Evidence for a role of the ubiquitin-proteasome system in signal transduction came from experiments of Verdier and co-workers who showed that a Cis member of the SOCS family was ubiquitinated upon erythropoietin receptor activation (19). Thus, at least two mechanisms for the termination of the GHR phosphorylation might depend on proteolysis: the regulation of phosphatases and of the SOCS proteins.

Until now there is clear evidence that tyrosine kinase receptors, like the epidermal growth factor and the insulin receptor, continue to signal after endocytosis (42, 43). Our data with the GHR show that initiation as well as termination of its phosphorylation as well as of JAK2 can occur at the cell-surface. No evidence is available about signal transduction inside the cells. Combining an acid wash procedure with anti-GH immunoprecipitation we show that the GHR can induce a second round of signal transduction intracellularly. This is not unexpected because obviously GH keeps the two GHRs complexed after endocytosis. In this configuration JAK2 has high affinity for the complex and will either rebind (if it was removed during passage of the coated pits) or will keep its position on the dimerized tails once internalized. Although the amount of undegraded, endocytosed wtGHR is very small, the western blot signal of JAK2 complexed to the GH-GHR complexes is significant as compared to control (non acid-washed) cells. This indicates that the signaling capacity of GH-GHR complexes in endosomes is significant. The relevance for signal transduction in endosomes is not clear. It is possible that the signaling GHR complexes in endosomes differ from those at the cell-surface. This is not obvious from our data, because the SDS-PAGE patterns of phosphotyrosine-containing proteins of total and endocytosed GH complexes look very similar. Together, these observations indicate that GHR signal transduction continues or resumes after endocytosis and that the signals, regenerated at the two cellular locations, do not substantially differ.

Another point of discussion is the presence of truncated GHR originating from the wtGHR, not from the truncated GHR 1-369. Firstly, it is not clear where this process starts. Experiments with the GHR F327A show that it is ubiquitin system (UbE motif) independent, because it also occurs in this mutant GHR, and in presence of proteasome inhibitors its formation can still occur (unpublished results). Thus, the GHR is C-terminally truncated already at the cell-surface and the truncated GHR can endocytose, complexed to GH. It remains to be determined, whether this truncated GHR plays a role in signal transduction.

Both JAK2 and a multitude of other, mostly high molecular weight, proteins are activated and interact with both wtGHR and GHR 1-369 after GH induction in acid-wash treated cells. Co-immunoprecipitation of GH-GHR-JAK2 complexes, after uptake by the cells, shows that JAK2 is not only bound to the GHR at the cell-surface but also intracellularly, suggesting that the receptor and some of its signal
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transducing molecules might still be active in endosomes. A smear of phosphorylated proteins attached to the GH-GHR complex inside the cells confirmed the receptor's capacity of signaling. Intracellularly, the pattern of phosphorylated proteins in wtGHR and GHR 1-369 is similar, providing both receptors comparable signaling capabilities.

Acknowledgements

We thank Jürgen Gent, Julia Schantl and Toine ten Broeke for creative discussions. This work was supported by grants of the Netherlands Organization for Scientific Research NWO-902-68-244 and by an European Union Network grant (ERBFM-RXCT96-0026).

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