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Differential Activation of Stat3 and Stat5 by Distinct Regions of the Growth Hormone Receptor

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The GH receptor (GHR) is a member of the cytokine receptor superfamily; its signaling involves the activation of Janus tyrosine kinases (JAK2) and Stat (signal transducers and activators of transcription) transcription factors. Using truncated and tyrosine mutants of the receptor, we show that different receptor domains are essential for the activation of Stat3 and Stat5. GH-dependent phosphorylation of JAK2, Stat3, and Stat5, as well as transactivation studies with reporter genes containing Stat3 and Stat5 DNA-binding elements, was performed in cells expressing the various GHR mutants. The membrane-proximal region of the receptor necessary for JAK2 activation is sufficient for Stat3 activation. In contrast, C-terminal tyrosine residues of GHR are absolutely required for Stat5 activation. The same residues are also involved in the regulation of JAK2 dephosphorylation, possibly through the activation of a phosphatase. Using *in vitro* experiments with glutathione-*S*-transferase-fusion proteins, we demonstrate that the SH2 domain of Stat5 binds to the carboxy-terminal tyrosine-phosphorylated residues of GHR. Our results show that a cytokine receptor can mediate differently the activation of distinct Stat proteins that could be involved in cytokine-specific effects. (Molecular Endocrinology 10: 998–1009, 1996)

INTRODUCTION

GH is a pituitary hormone that promotes growth and exerts a large number of metabolic effects. The GH receptor (GHR) is a single transmembrane protein that belongs to the cytokine receptor superfamily (1, 2). These receptors have conserved structural features, including paired cysteine residues and a WSXWS motif in the extracellular domain; a conserved proline-rich region (box 1) is also found in the cytoplasmic domain of most members of the superfamily. While they have no intrinsic enzymatic activity, these receptors associate with and activate members of the Janus kinase (JAK) family (reviewed in Ref. 3). GHR signaling involves ligand-induced homodimerization of the receptor and its association with the tyrosine kinase JAK2 (4); these events initiate a cascade of phosphorylation of cellular proteins, including the kinase itself and the receptor. Stat proteins (signal transducers and activators of transcription) have been shown to participate in cytokine receptor signaling. Six members of the Stat family have been identified to date. Among them, Stat1, Stat3, and Stat5 are involved in many receptor-signaling pathways (reviewed in Ref. 5) including those of GHR (6–8). Genetic and biochemical evidence has demonstrated that Stat proteins are targets of JAK kinases (reviewed in Ref. 9). The mechanism proposed for the activation of these transcription factors involves their tyrosine phosphorylation by the receptor-associated kinases and translocation to the nucleus, where they bind specific DNA sequence motifs, thus contributing to transcriptional activation of cytokine-regulated genes. In addition, it has been reported recently that serine/theonine phosphorylation of some Stat proteins could be important in the regulation of their activity (10, 11).

GH regulates the expression of many genes involved in proliferation and differentiation (reviewed in Ref. 12). Among the GH-regulated genes, the serine protease inhibitor, Spi 2.1, has been well characterized (13). The promoter of Spi 2.1 contains a short palindromic sequence that binds Stat5 (8). GH also activates transcription of the c-*fos* gene (14), and we have shown that GH induces binding of Stat3 to the c-*sis*-inducible element (SIE) of the c-*fos* promoter (15). We have previously characterized the regions of the re-

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ceptor involved in GH-induced gene transcription. The membrane-proximal domain, containing box 1, is involved in JAK2 activation but is not sufficient for GH-stimulated transcription of Spi 2.1. Additional C-terminal domains of the receptor are important for this effect (16). We have also demonstrated that Stat3 DNA-binding activity induced by GH does not require the carboxy-terminal region of GHR (15).

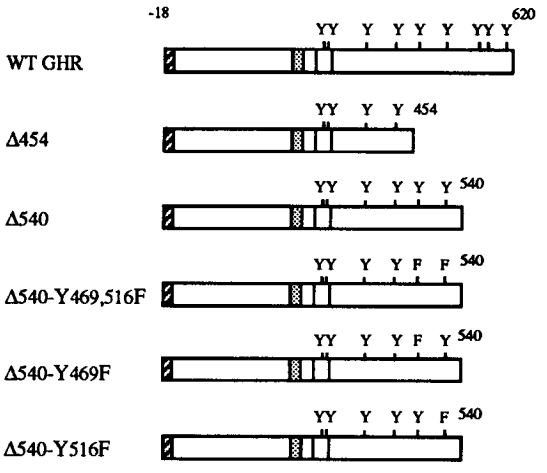
In this paper, we focus on regions of the receptor regulating the JAK/Stat pathways to better understand the participation of these proteins in GH-induced transcription. Several truncated and tyrosine-substituted mutant forms of GHR were constructed. In cells expressing the various GHR mutants, tyrosine phosphorylation kinetics of JAK2 were analyzed, and the activation of Stat3 and Stat5 were measured. For this purpose we developed a functional approach. DNA elements that have been reported to bind Stat proteins and that are present in GH-responsive gene promoters were used to construct reporter genes. Stat3-mediated transcription was measured using the SIE element of the c-*fos* promoter. Stat5-mediated transcription was evaluated with lactogenic hormone response element (LHRE) and GH response element II (GHREII), which are found in the β-casein and Spi 2.1 gene promoters, respectively. The use of these regulatory elements, rather than the full-length promoters, allows measurement of transcription mediated primarily by Stat3 and Stat5 and evaluation of the contribution of Stat proteins in the regulation of the transcription of the full-length promoter by GH. We identify two carboxy-terminal tyrosine residues important for GHR signaling. These tyrosines probably recruit a tyrosine phosphatase that participates in JAK2 dephosphorylation. The same tyrosines are important for the GH-induced tyrosine phosphorylation and the transcriptional activation of Stat5 but are not essential for Stat3 activation. Using an *in vitro* approach with glutathion-*S*-transferase (GST)-fusion proteins, we demonstrate the recruitment of Stat5 by the C-terminal region of GHR through an interaction between the SH2 domain of Stat5 and the phosphorylated tyrosine residues of the receptor. These data strongly suggest that separate regions of the GHR can mediate the recruitment of different Stat proteins regulating distinct signaling pathways.

RESULTS

Characterization of Stable Chinese Hamster Ovary (CHO) Clones Expressing Mutant Forms of GHR

To analyze the functional domains of GHR involved in the JAK/Stat pathways, we designed several mutant forms of the receptor (Fig. 1A). Two carboxy-terminal deletion mutants lacking the last 80 (Δ540) and 166 residues (Δ454), respectively, were constructed. Two

A.



B.

GH receptor form	Kd (nM)	Sites/cell
WT	0.45	90,000
Δ454	0.35	110,000
Δ540	0.33	100,000
Δ540-Y469,516F	0.12	110,000

Fig. 1. Construction and Expression of Mutated Forms of Rabbit GHR

A, Schematic representation of wild type (WT) and mutated forms of rabbit GHR. Mutant forms of the receptor are shown *below* the WT; *numbers* indicate amino acid (aa) residues. Residue 1 is the first aa of the mature protein. Boxes 1 and 2 are indicated as *solid lines*, the signal peptide sequence as a *hatched rectangle*, and the transmembrane domain as a *stippled rectangle*. The tyrosine residues substituted with phenylalanine are indicated. B, GH-binding properties of stable CHO clones expressing WT or mutant GHR. The binding of [^{125}I]hGH to intact cells was measured in the presence of increasing concentrations of unlabeled hGH. Dissociation constants (K_d) and receptor numbers (sites per cell) were calculated by Scatchard analysis.

tyrosines are present in the cytoplasmic domain of the rabbit GHR unique to Δ540, at positions 469 and 516. To study the importance of these tyrosine residues in GHR signaling, mutants were constructed in which tyrosines 469 and/or 516 were substituted with phenylalanine in Δ540 GHR.

CHO cells were stably transfected with wild type (WT), Δ454, Δ540, and Δ540-Y469,516F forms of GHR. Binding studies and Scatchard analysis (Fig. 1B) indicate that all mutant receptors are expressed at the cell surface with a comparable number of sites and show the similar binding affinities for human (h) GH. The size of the receptors and their ability to become tyrosine phosphorylated after GH stimulation were determined in different CHO clones stimulated with bi-

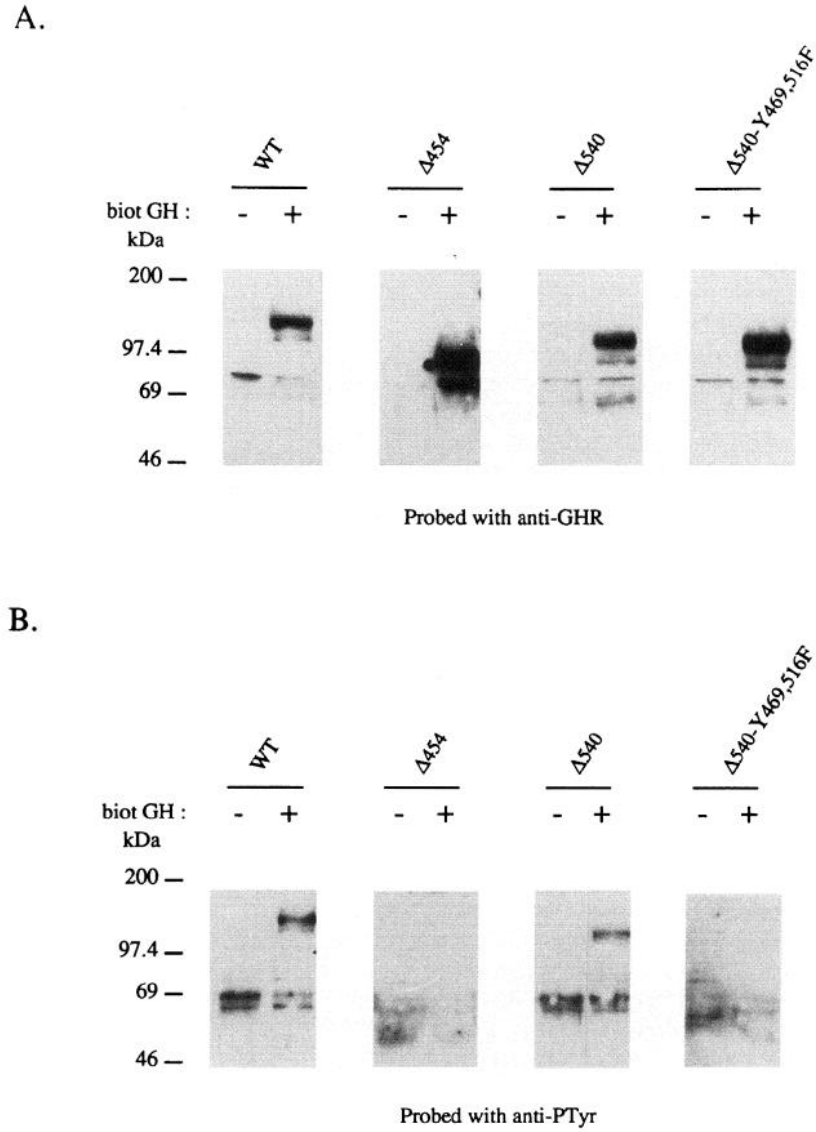


Fig. 2. Tyrosine Phosphorylation of WT and Mutant Forms of GHR

Stable CHO clones were stimulated (+) or not (−) with 20 nM biotinylated hGH for 5 min, and the GH/GHR complexes were purified using streptavidin agarose. Western blot analysis was performed with anti-GHR antibody (A) or with anti-PTyr antibody (B). The molecular masses are indicated on the *left*.

otinylated hGH. The complexes formed were precipitated with streptavidin beads and analyzed by Western blot. Immunoblotting with an anti-GHR antibody revealed molecular masses for the WT, Δ454, Δ540, and Δ540-Y469,516F receptors, of 120, 95, 110, and 110 kDa, respectively (Fig. 2A). The smaller molecular mass bands probably correspond to degradation products of GHR. Western blot analysis using anti-PTyr antibody detected phosphorylated proteins only in cell lines expressing WT and Δ540 receptors (Fig. 2B). These bands correspond in size to the WT and Δ540 receptors, suggesting their identity. In the other stable clones, we did not detect any phosphorylated proteins. We therefore conclude that tyrosine residues 469 and 516 are two major phosphorylation sites of GHR.

The C-Terminal Tyrosine Residues of GHR Are Involved in JAK2 Dephosphorylation

The activation of the associated tyrosine kinase JAK2 is one of the first steps of GHR signal transduction. As JAK kinases phosphorylate Stat proteins, the study of JAK2 phosphorylation is important for the understanding of the JAK/Stat signaling by GHR. We compared the kinetics of phosphorylation of JAK2 in the various stable clones expressing the mutant forms of GHR (Fig. 3A). The amount of immunoprecipitated JAK2 in each lane was evaluated by reprobing the membrane with anti-JAK2 antibody. The amount of phosphorylated JAK2 *vs.* the amount of immunoprecipitated JAK2 was quan-

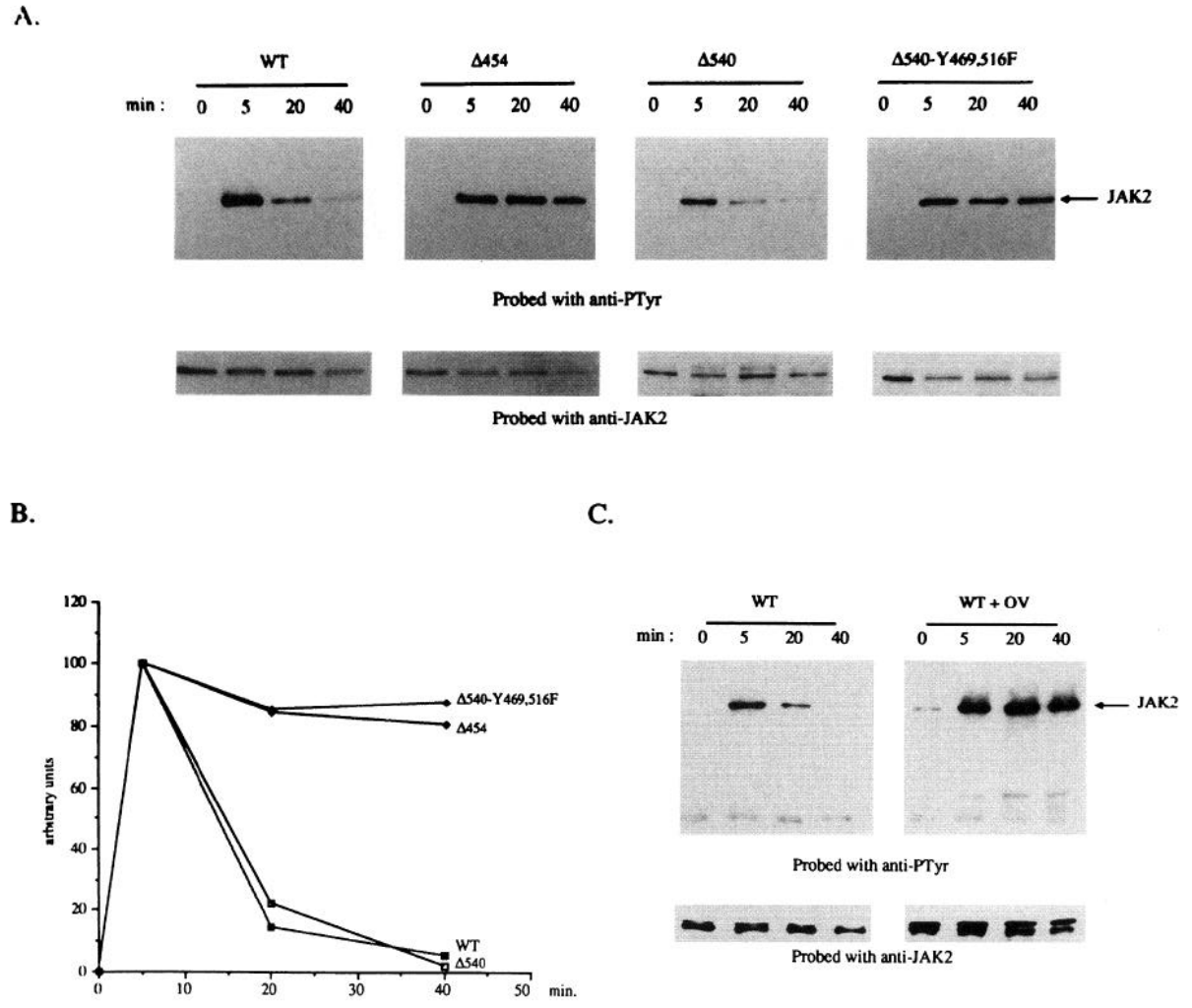


Fig. 3. Kinetics of Tyrosine Phosphorylation of JAK2 in CHO Cells Expressing WT and Mutant Forms of GHR

A, Cells were stimulated with 20 nM hGH for 0, 5, 20, or 40 min. Whole cell lysates were immunoprecipitated with anti-JAK2 antibody and analyzed by Western blot with anti-PTyr or anti-JAK2 antibody. B, Quantification of the relative extent of JAK2 phosphorylation after GH stimulation. The amount of tyrosine-phosphorylated JAK2, as assayed by densitometric scanning (arbitrary units), was normalized to the similarly quantitated amount of JAK2 recovered in each precipitate. C, Effect of orthovanadate on JAK2 phosphorylation. CHO cells expressing the WT receptor were preincubated for 1 h with 1 mM orthovanadate (OV) and then were treated as described in panel A.

tified using densitometry; the results are shown in Fig. 3B. In cell lines expressing WT and Δ540 receptors, tyrosine phosphorylation of JAK2 is transient with a peak at 5 min of stimulation after which phosphorylation declines. In contrast, in cell lines expressing Δ454 and Δ540-Y469,516F, phosphorylation of JAK2 is persistent, and no decrease is observed even at 40 min. These observations strongly suggest that tyrosine residues 469 and/or 516 are involved in the recruitment or the activation of a tyrosine phosphatase. To support this hypothesis, cells expressing WT receptors were preincubated with orthovanadate for 1 h before the analysis of JAK2 phosphorylation (Fig. 3C). Under such conditions, the tyrosine phosphorylation of JAK2 was also sustained.

The C-Terminal Domain of GHR Is Not Essential for Stat3 Activation by GH

To study the activation of Stat3 by GH, two different approaches were used. Since tyrosine phosphorylation of Stat proteins is essential for their activity, we first analyzed the GH-induced phosphorylation of Stat3. A functional assay was then developed to measure the transcriptional activity mediated by Stat3 under GH stimulation. For this purpose, a reporter gene containing three copies of m67 (high-affinity mutated form of SIE) coupled to the thymidine kinase (TK) minimal promoter and the luciferase gene was constructed.

GH-induced tyrosine phosphorylation of Stat3 was analyzed in CHO cell lines stably expressing the WT or

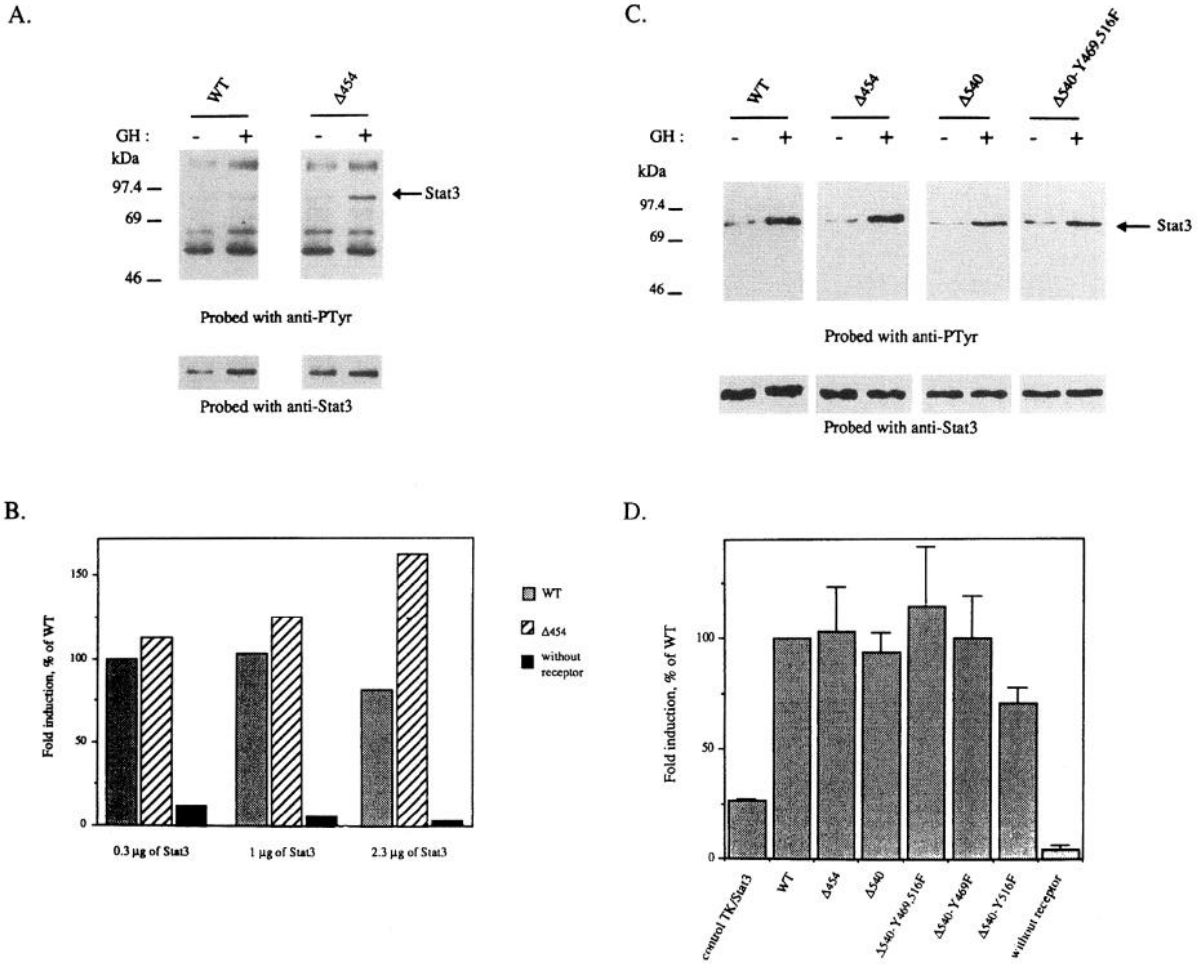


Fig. 4. GH-Induced Tyrosine Phosphorylation and Transcriptional Activity of Stat3 in CHO and 293 Cells Expressing WT and Mutant Forms of GHR

A, Stable CHO clones expressing WT or Δ454 receptor were stimulated (+) or not (−) with 20 nM hGH for 15 min, and whole cell extracts were immunoprecipitated with anti-Stat3 antibody. Western blot analysis was performed with anti-PTyr or anti-Stat3 antibody. Molecular masses of the protein standards are indicated on the *left*. The *arrow* indicates the migration of endogenous Stat3 protein. B, CHO clones cotransfected with m67-TK-luc, different amounts (0.3–2.3 μg) of Stat3 expression vector, and pCH110 were incubated in the presence of 50 nM hGH and 250 nM dexamethasone or 250 nM dexamethasone alone. Cell extracts were prepared and assayed for luciferase and β-galactosidase activities, and the values for luciferase activity were normalized to β-galactosidase activity for each transfection experiment. The fold induction was calculated as normalized luciferase activity in the presence of both hormones divided by luciferase activity in the presence of dexamethasone alone. Results of a representative experiment are expressed as percent of WT. C, 293 cells were cotransfected with 2 μg GHR, 2 μg Stat3, and 0.05 μg JAK2 expression vectors. Forty eight hours after transfection, cells were stimulated (+) or not (−) with 20 nM hGH for 15 min. Cell lysates were immunoprecipitated with anti-Stat3 antibody and then subjected to Western blot analysis using anti-PTyr or anti-Stat3 antibody. The *arrow* indicates the migration of Stat3 protein. D, Stat3-mediated transcriptional activation induced by GH in 293 cells expressing WT and mutant forms of GHR. 293 cells cotransfected with GHR and Stat3 expression vectors, m67-TK-luc reporter gene, and pCH110 were incubated in the presence of 50 nM hGH and 250 nM dexamethasone or 250 nM dexamethasone alone. Control transfections were performed without receptor expression vector or using TK-luc reporter gene. Cell extracts were prepared and assayed for luciferase and β-galactosidase activities; the values for luciferase activity were normalized to β-galactosidase activity for each transfection experiment. The fold induction was calculated as normalized luciferase activity in the presence of both hormones divided by luciferase activity in the presence of dexamethasone alone. Results are expressed as percent of WT and represent the mean (SEM) of three to five independent experiments.

Δ454 receptors. After GH stimulation, cell lysates were immunoprecipitated with anti-Stat3 antibody, and Western blot analysis was performed with anti-PTyr or anti-Stat3. In CHO cells expressing Δ454 receptor, a GH-induced tyrosine phosphorylation of endogenous Stat3 was detected. In contrast, we failed to detect any induced phosphorylation of Stat3 in CHO cells expressing WT receptor (Fig. 4A), suggesting that the amount of phosphorylated endogenous Stat3 was below the detection limit of the antibodies used. In the same cells, transcriptional studies revealed that both WT and Δ454 receptors are able to activate additional

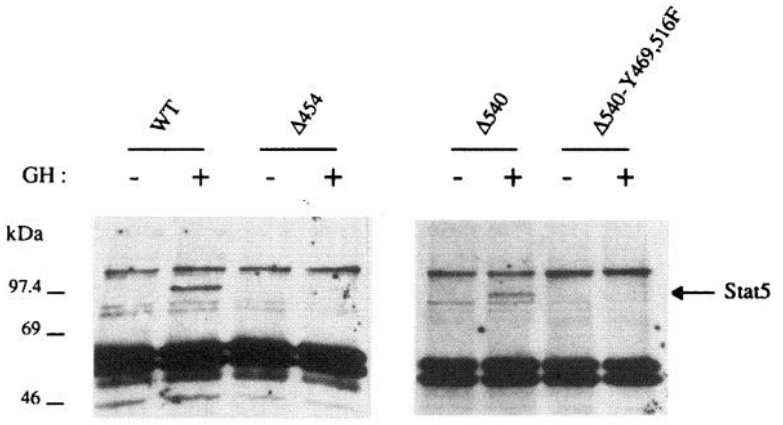


Fig. 5. Tyrosine Phosphorylation of Endogenous Stat5 Induced by GH in CHO Cells Expressing WT or Mutant Forms of GHR
Cells were stimulated (+) or not (−) with 20 nM hGH for 15 min, and whole cell extracts were immunoprecipitated with anti-PTyr antibody. Western blot analysis was performed with anti-Stat5 antibody. Molecular masses of the protein standards are indicated on the *left*. The *arrow* indicates the migration of Stat5 protein.

exogenous Stat3. Moreover, when 2.3 μg Stat3 cDNA were transfected, the transcriptional activity measured in cells expressing Δ454 receptor was significantly higher (153 ± 7.3% of WT, three independent experiments) (Fig. 4B). The greater phosphorylation and activation of Stat3 in CHO cells expressing Δ454 receptor is consistent with the increased DNA-binding activity of Stat3 previously observed (15) and could be correlated to the sustained activation of JAK2 or to the absence of a negative regulatory domain within the receptor.

To detect a Stat3 tyrosine phosphorylation signal for the WT receptor, we used a transient overexpression cellular system consisting of 293 cells transfected with the various forms of GHR, Stat3, and JAK2 expression vectors. A similar level of expression of the different receptor forms at the cell surface was checked by binding experiments (data not shown). After GH stimulation for 15 min, cell lysates were immunoprecipitated with anti-Stat3 antibody, and Western blot analysis was performed with anti-PTyr or anti-Stat3. In cells expressing either of four forms of GHR, a clear induction of Stat3 tyrosine phosphorylation was detected upon GH stimulation (Fig. 4C, *upper panel*). The amount of Stat3 protein present in the lysates from stimulated or unstimulated cells was equivalent as determined after reprobing the membrane with an anti-Stat3 antibody (Fig. 4C, *lower panel*). Transcriptional studies carried out in 293 cells transfected with cDNAs encoding GHR forms, Stat3, and the reporter construct (Fig. 4D) showed a significant GH-dependent transcriptional activation with all receptors (100% representing 5-fold induction for the WT receptor). In contrast, little or no activation was measured without receptor or when a control TK-luciferase reporter gene was used. The overexpression system and the sensitivity of the reporter construct used can explain the similar results obtained for WT and Δ454 receptors in 293 cells in comparison to the observations in the CHO system.

Taken together, these results show that the C-terminal portion (residues 455–620) of the GHR is not essential for GH-induced tyrosine phosphorylation and transcriptional activation of Stat3.

The C-Terminal Tyrosine Residues of Rabbit GHR Are Required in Stat5 Activation by GH

We studied the GH-dependent tyrosine phosphorylation of Stat5 and its functional activation. To evaluate the transcriptional activity mediated by Stat5, two reporter genes were constructed. In one, the LHRE was fused to the TK promoter and the luciferase gene. LHRE is an element of the β-casein promoter that was used for the affinity purification of Stat5 (17). In the second reporter gene, the GHREII was fused to TK and luciferase. GHREII is a GH-regulated element of the Spi 2.1 promoter that binds Stat5 (8). Using this approach, we were able to obtain information on the role of Stat5 in the transcriptional regulation of genes such as Spi 2.1 and β-casein.

We analyzed the ability of GH to induce phosphorylation of endogenous Stat5 in CHO cell lines stably expressing the WT and the mutant forms of the receptor. Immunoprecipitation with anti-PTyr antibody followed by Western blot analysis with anti-Stat5 antibody were performed. As shown in Fig. 5, GH-induced phosphorylation of Stat5 is detected only in cells expressing WT or Δ540 receptor. No phosphorylation of Stat5 was detected in cells expressing Δ454 or Δ540-Y469,516F receptor, suggesting that the receptor domain between residues 455 and 540 (in particular tyrosines 469 and/or 516) is important for Stat5 tyrosine phosphorylation upon GH stimulation.

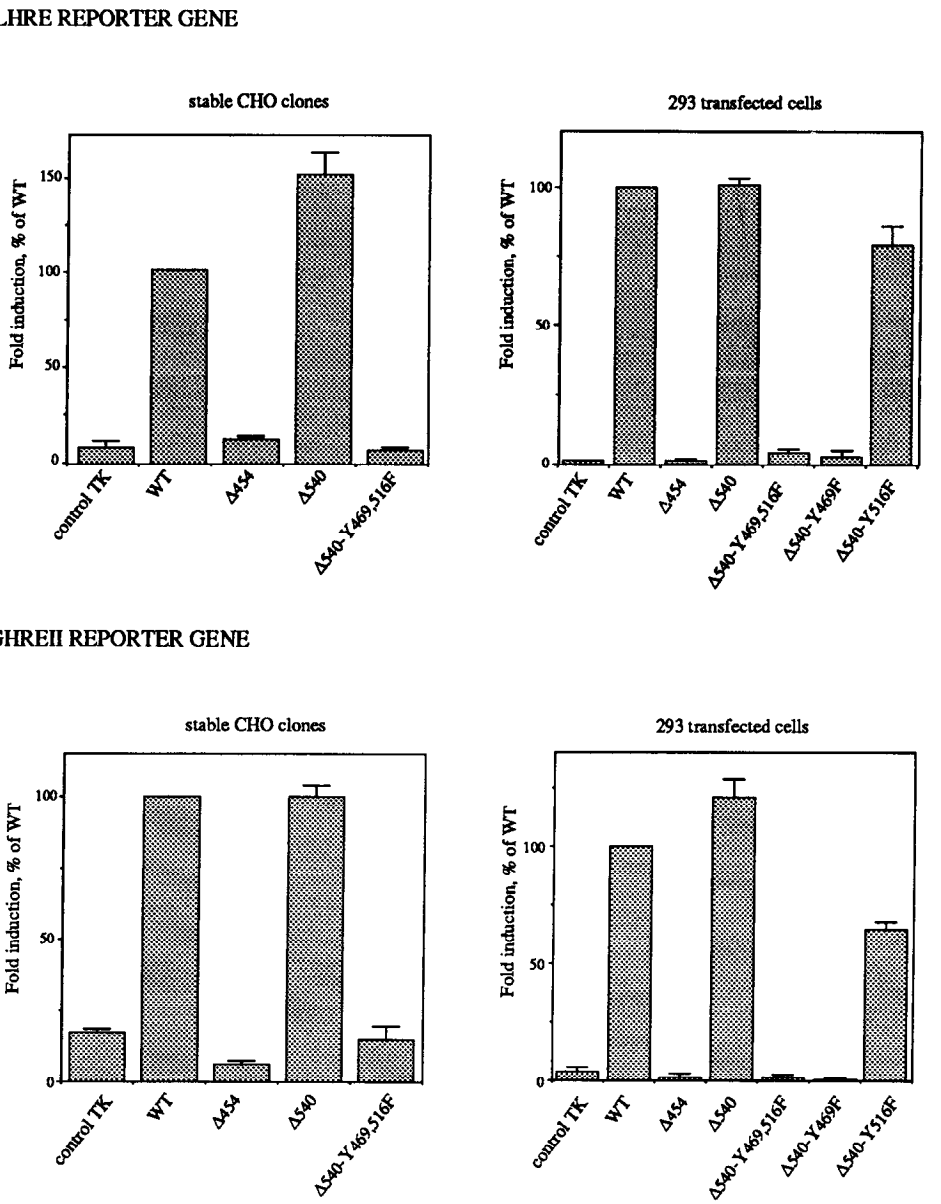


Fig. 6. Stat5-Mediated Transcriptional Activation Induced by GH in CHO or 293 Cells Expressing WT or Mutant Forms of GHR

Cells cotransfected with GHR cDNA (for 293 cells only), LHRE-TK-luc (A), or GHREII-TK-luc (B) reporter gene and pCH110 were incubated in the presence of 50 nM hGH and 250 nM dexamethasone or 250 nM dexamethasone alone. Control transfections were performed using TK-luc reporter gene. Cell extracts were prepared and assayed for luciferase and β-galactosidase activities, and the values for luciferase activity were normalized to β-galactosidase activity for each transfection experiment. The fold induction was calculated as normalized luciferase activity in the presence of both hormones divided by luciferase activity in the presence of dexamethasone alone. Results are expressed as percent of WT and represent the mean (SEM) of three to five independent experiments.

GH-induced transcriptional activation of both reporter constructs (LHRE and GHREII) was measured in two different cellular systems. Stable CHO clones expressing the various forms of GHR were transfected with the two reporter genes; 293 cells were transiently cotransfected with the GHR mutant expression vectors and the reporter constructs. As shown in Fig. 6, transcriptional activation through LHRE or GHREII is observed in CHO cells expressing WT or Δ540 receptor (100% representing 8-fold induction for the WT receptor with LHRE reporter gene and 5-fold induction with GHREII reporter gene) but not in CHO cells expressing Δ454 or Δ540-Y469,516F receptor. Similar results were observed in transfected 293 cells (100% representing 13-fold induction for the WT receptor with LHRE reporter gene and 7-fold induction with GHREII reporter gene). Moreover, the single tyrosine mutant Δ540-Y469F failed to transactivate either reporter construct in 293 cells, while the Δ540-Y516F mutant was able to induce transcription. These results

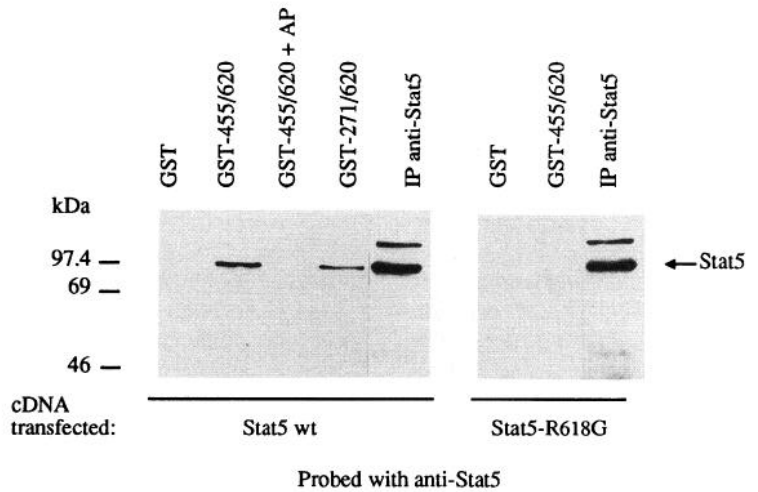


Fig. 7. *In Vitro* Studies

Association of Stat5 with the C-terminal-phosphorylated residues of GHR. 293 cells were transfected with wild type Stat5 (wt) or Stat5-R618G expression vectors. Cell lysates were incubated with phosphorylated GST-fusion proteins containing the C-terminal region of GHR (GST-455/620) or the entire intracellular domain (GST-271/620) or with GST-455/620 protein dephosphorylated by alkaline phosphatase (GST-455/620 + AP). GST protein was used as a control. To control the amount of Stat5 wt or Stat5-R-618G expressed, cell lysates were immunoprecipitated with anti-Stat5 antibody. The complexes formed were then analyzed by Western blot with anti-Stat5 antibody. The molecular masses of the protein standards are indicated on the *left*. The *arrow* indicates the migration of Stat5 proteins.

demonstrate that, in the context of Δ540 GHR, tyrosine residue 469 is required for Stat5-mediated transcription induced by GH.

The SH2 Domain of Stat5 Binds the C-Terminal Phosphorylated Tyrosines of GHR

An *in vitro* approach was developed to study the putative interactions between Stat3, Stat5, and the cytoplasmic residues of the GHR. Different GST-fusion proteins containing the C-terminal region of GHR, from residues 455–620 (GST-455/620), the entire cytoplasmic domain of the receptor (GST-271/620), or GST alone, were produced in bacteria in which phosphorylation of tyrosines occurs. Lysates of 293 cells, transfected with Stat5 or Stat3 expression vectors, were incubated with 2 μg of the various GST fusion proteins attached to glutathione beads. Western blot analyses using anti-Stat5 or anti-Stat3 antibodies were performed to detect the association of Stat5 or Stat3 with the GST fusion proteins.

Stat5 was detected on GST-455/620 beads, but not on GST beads (Fig. 7), showing a specific interaction of Stat5 with the C-terminal region of the receptor. After pretreatment of the GST-455/620 beads with alkaline phosphatase, no Stat5 was detected, suggesting the involvement of phosphorylated tyrosine residues of the receptor in this interaction. As expected, Stat5 also associated with the entire intracellular domain of the GHR (GST-271/620).

Transfection experiments of 293 cells with an expression vector encoding a point mutant of Stat5 in which the conserved arginine residue of the SH2 domain was changed into glycine (Stat5-R618G) were performed, and, in this case, no interaction with GST-455/620-phosphorylated fusion protein was detected. We verified that both wild type Stat5 and Stat5-R618G were expressed at comparable levels: the same amount of protein was detected by Western blot using anti-Stat5 antibody after immunoprecipitation with anti-Stat5 in cells transfected with both cDNAs. The higher molecular mass band represents a nonspecific interaction of the antibodies used.

Taken together, these data demonstrate that the Stat5/GHR interaction is mediated by the SH2 domain of Stat5 and the phosphorylated tyrosines of the receptor.

Using the same approach, we were not able to detect any specific interaction between Stat3 and the cytoplasmic domain of GHR: Stat3 bound to the GST protein alone, as well as to GST-455/620 and GST-271/620 proteins (data not shown).

DISCUSSION

JAK kinases and Stat transcription factors have been shown to participate in the intracellular signaling of cytokine receptors. In particular, GHR associates with the JAK2 tyrosine kinase and activates Stat1, Stat3, and Stat5 transcription factors. Genetic experiments or analysis of genetic defects in humans (18–20), as well as the studies with dominant negative mutants of JAK kinases (21), suggest that most pathways induced by cytokine receptors are dependent on JAK kinases. Among these pathways, the involvement of JAK tyrosine kinases in Stat protein activation has been well

established. The use of mutant forms of cytokine receptors allowed identification of regions important for JAK kinase activation that were absolutely essential for Stat activation (22, 23). Furthermore, *in vitro* experiments directly demonstrated a tyrosine phosphorylation of *in vitro* transcribed and translated Stat5 by JAK2 kinase (24). However, the role of Stat proteins in cytokine-induced responses is not completely understood. In some cases, the activation of Stat proteins could be linked with ligand-induced proliferation and, in other cases, there is a divergence between Stat activation and proliferation. For example, the membrane-proximal region of the β_c-chain of the interleukin-3 receptor is sufficient for Stat5 activation and DNA synthesis (25). In contrast, the C-terminal region of the interleukin-2 receptor is necessary for Stat5 activation but not for ligand-induced proliferation (26).

We and others have identified different functional regions in the cytoplasmic domain of GHR. The membrane-proximal region of the receptor, containing the proline-rich conserved motif (Box 1), is essential and sufficient for GH-induced activation of JAK2, proliferation, and activation of some Stat proteins (22, 27, 28). In contrast, transcriptional activation of GH-induced genes, including Spi 2.1 (16, 29) and the insulin gene (30), requires additional C-terminal sequences of the GHR. Many GH-responsive promoters and, in particular, those of Spi 2.1, β-casein, and c-*fos* genes, contain Stat-binding sequences. To characterize the molecules and the residues of the receptor involved in GH-induced transcription, we have analyzed the JAK/Stat pathways. In preliminary studies, two C-terminal regions (from residues 455–540 and 541–620) were found to be involved in transcriptional activation of Spi 2.1 or β-casein promoter by GH (our unpublished data). In the present paper, we focused on one of these functional domains: the cytoplasmic region between residues 455 and 540. As tyrosine residues can be essential for the recruitment of SH2-containing signaling molecules, the two tyrosines present in this region (Y469 and Y516) were mutated to phenylalanine. As shown in the experiments using biotinylated GH, one or both of these tyrosines are phosphorylated after GH stimulation and constitute major sites of phosphorylation.

The phosphorylation of JAK2 kinase was analyzed in several cell lines expressing WT or mutant forms of GHR. Our results suggest that tyrosine residues 469 and/or 516 of GHR are involved in the activation of a tyrosine phosphatase that could dephosphorylate JAK2 and permit the down-regulation of the GH response in cells. In the same cellular system, a low level of JAK1 phosphorylation induced by GH was detected, and the C-terminal region of the receptor seemed to be involved in JAK1 down-regulation as well (our unpublished data). For several members of the cytokine receptor superfamily (erythropoietin, interleukin-3, interferon α/β receptors), phosphatase molecules have been implicated in their signaling pathways. Recently, phosphatase PTP1D has been reported to be recruited by the PRL receptor and to positively regulate gene-specific transcription (34). The identification of the phosphatase involved in GH signaling is currently under investigation. The involvement of tyrosine phosphatase in GHR signaling could participate in the regulation of Stat protein activity by modulating JAK kinase phosphorylation and activation.

To study the activation of Stat transcription factors by GH, we developed a functional approach that allows us to analyze the transcriptional activity of Stat proteins other than, and not limited to, their ability to bind a DNA target sequence as in gel shift experiments. The results obtained with these reporter gene constructs clearly showed that different regions of the receptor are required for the GH-induced activation of Stat3 and Stat5.

In previous studies, we have shown that GH induces DNA-binding activity of Stat3 in CHO cells expressing C-terminal-deleted receptors (from residues 455–620) (15). We now present evidence that this region is essential neither for tyrosine phosphorylation of Stat3 nor for Stat3-mediated transcription induced by GH. For other cytokine receptors, it has been reported that Stat3 is recruited via tyrosine residues of their cytoplasmic domain, and the consensus sequence YXXQ present in gp130 has been proposed as a specific motif for Stat3 recruitment (35). This motif is not present in the GHR. Mutants of granulocyte colony-stimulating factor receptor lacking this particular sequence are still able to activate Stat3 (23). Here, we demonstrate that the carboxy-terminal tyrosines (469 and 516) are not required for Stat3 activation. Results from other groups showed that a truncated GHR mutant, lacking all cytoplasmic tyrosine residues, was still able to activate Stat-binding activity to SIE and γ-responsive region probes that essentially bind Stat1 or Stat3 (36, 37). Taken together, these observations suggest that tyrosine residues of GHR are not essential for the activation of some Stat proteins and, in particular, Stat3. However, the consensus motif (YXXQ) is present in the sequence of JAK2, and we propose that Stat3 could be recruited by this GHR-associated kinase. This model is supported by recent results of Gupta *et al.* (38) showing that Stat1 and Stat2 could directly interact with JAK kinases. Such a hypothesis could explain the higher levels of Stat3 tyrosine phosphorylation and transcriptional activation observed in the CHO cell lines expressing Δ454. This could be due in part to the sustained phosphorylation of JAK2 and would be consistent with a possible enhancement of Stat3 recruitment by JAK2. However, although the use of truncation mutants of GHR suggests that the C-terminal region of GHR is not essential for Stat3 activation, a putative interaction of Stat3 with this region cannot be excluded in the context of the full-length receptor.

In contrast, the C-terminal residues (455–540) of GHR are essential for Stat5 activation. Phosphorylation studies in stable CHO clones showed that ty-

rosines 469 and/or 516 of the receptor were required for GH-induced phosphorylation of Stat5. As one or both tyrosines are phosphorylated upon GH stimulation, they could be important for the recruitment of Stat5 by GHR via the Stat5 SH2 domain. Using two reporter genes containing the LHRE and GHREII elements, GH-induced transcription was observed in two cellular systems (CHO and 293 cells) expressing the full-length GHR. It has been reported that these two elements are able to bind only Stat5 and no other Stat proteins (8, 39); our results thus show that Stat5 is transcriptionally functional after GH stimulation. In COS cells transfected with GHR cDNA and a reporter construct containing a part of the β-casein promoter including a single Stat5 binding site, GH was able to induce DNA binding, but not transcriptional activity, of Stat5 (40). Differences in the cellular system and/or in the reporter gene could explain this discrepancy. The absence of Stat5-mediated transcriptional activity with Δ454 and Δ540-Y469,516F mutants indicates a correlation between Stat5 phosphorylation and transcriptional activity. More precisely, we identified the specific tyrosine residue (tyrosine 469) essential for Stat5 activation in the context of Δ540 GHR. This tyrosine is probably involved in Stat5 recruitment. A cytoplasmic region of porcine GHR, containing the tyrosine residue homologous to Y469 in rabbit GHR, has been shown to be important for the GH-induced tyrosine phosphorylation of a 95-kDa protein that could be Stat5 (41). In addition, since mutants Δ454, Δ540-Y469,516F, and Δ540-Y469F of GHR were also unable to induce the transcription of the full-length promoters of Spi or β-casein genes upon GH stimulation (our unpublished data), a correlation can be established between the noninducibility of Spi or β-casein transcription and the lack of activation of Stat5 by these mutant forms. Our data strongly suggest that Stat5 is crucial and absolutely required for both Spi and β-casein gene transcription induced by GH.

In vitro experiments demonstrate an interaction between Stat5 and the phosphorylated tyrosines of the C-terminal domain of the receptor (GST-455/620). In addition, we clearly show that this association takes place between the SH2 domain of Stat5 and carboxy-terminal phosphorylated tyrosines of GHR. For this purpose, a mutated form of Stat5 was used that contains a point mutation changing the absolutely conserved arginine in the floor of the SH2 phosphotyrosyl pocket to glycine.

Taken together, our data demonstrate that different domains of the GHR are essential in Stat3 and Stat5 recruitment and activation: the membrane-proximal region is sufficient for Stat3 activation, possibly because of the recruitment of Stat3 by JAK2, and the GHR C-terminal region is absolutely required for Stat5 recruitment through its tyrosine residues, leading to Stat5 activation. It has been shown that GH induces a short-term proliferation of BAF3 cells expressing mutant forms of GHR lacking the C-terminal tail (Ref. 22 and our unpublished data). In addition, transcription of GH-specific genes (Spi 2.1 and β-casein) requires the carboxy-terminal region of the receptor. Thus, we suggest that Stat3 may be involved in proliferation pathways, whereas Stat5 could play a role in differentiation pathways and in gene-specific transcription.

MATERIALS AND METHODS

Plasmids

LHRE, GHREII, and m67 reporter genes were constructed using a Puc 18 vector containing the TK minimal promoter linked to luciferase reporter gene (TK-luc) (42). Double-stranded oligonucleotides, 5′-CTGCAGTCGACATTTCCCGTAAATCGTC GACTGCA-3′ for m67 (three copies) and 5′-CTGCAGTGTGGACTTCTTGGAATTAA GGGACTTTTGCTGCAG-3′ for LHRE (six copies) were inserted into the *Pst*I site of the vector. Three copies of GHREII (13) were subcloned between *Sal*I/*Bam*HI of the vector.

The cDNA encoding the Δ454-truncated mutant of rabbit GHR was previously described (16). The cDNA encoding Δ540 was constructed by PCR, using primers that permit the addition of an in-frame stop codon and was inserted in pcDNA3 expression vector (Invitrogen, San Diego, CA). Mutagenesis on single-stranded DNA was done by the method of Kunkel using the following oligonucleotides: 5′-ACTAACCTGGGCGAAAAAGTCGATGTTTGC-3′ for the substitution of tyrosine 469 to phenylalanine and 5′-CTGCTTCACAGAAGA-AGGCGTTGTCCATG-3′ for the substitution of tyrosine 516 with phenylalanine.

The sequence encoding the C-terminal region of rabbit GHR (residue 455–620) was amplified by PCR and subcloned in the *Bam*HI site of pGEX-2TK (Pharmacia, Piscataway, NJ). The cDNA sequence encoding the entire cytoplasmic domain of GHR was cloned in pGEX-2T (Pharmacia) as described (43). The cDNA encoding sheep Stat5-R618G (subcloned in the expression vector pXM) was constructed by mutagenesis on single-stranded DNA using the following oligonucléotide: 5′-TTGTTGGGCTT TAGCGACTCA-3′.

Cell Culture/Purification of GHR Complexes on Streptavin-Agarose/Immunoprecipitation

Generation of stable CHO clones, transfection of 293 cells, binding assays, and cell culture conditions were performed as described (15, 16).

CHO (20×10^6) cells were cultured in serum-free medium overnight and incubated with or without 20 nM biotinylated hGH for 5 min at 37 C. Cell proteins were extracted in 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM Na orthovanadate, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride). Lysates were treated with steptavidin agarose as described (16) and subjected to 7.5% SDS-PAGE.

CHO (20×10^6) or 293 (10×10^6) cells (transfected with 2 μg GHR, 2 μg Stat3, and 0.05 μg JAK2 expression vectors) were cultured in serum-free medium overnight and stimulated with 20 nM hGH (kindly provided by Serono) for various time periods. Cells were lysed in 0.5 ml lysis buffer and incubated with the designated antibody (1 μg anti-PTyr (4G10, UBI), 1/500 of anti-JAK2 (UBI) or 1 μg anti-Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA). After treatment with protein A-Sepharose, lysates were subjected to 7.5% SDS-PAGE.

Western Blot

After separation by gel electrophoresis, proteins were transferred to nitrocellulose and subjected to immunoblot analysis. Membranes were incubated with the indicated antibody (0.1 μg/ml anti-Ptyr (4G10, UBI, Lake Placid, NY), 1/4000 of anti-JAK2 (UBI), 1/250 of anti-Stat5 (Transduction Laboratories, Lexington, KY), 0.2 μg/ml anti-Stat3, or 0.5 μg/ml anti-GHR (42) as described (16). Proteins were visualized using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Membranes were dehybridized of probes overnight at 4 C in an acid solution (0.1 M glycine, pH 3, 0.1 M NaCl) and reprobed with the indicated antibody.

Transcription Assays

Stable CHO clones were plated in six-well plates (10^6 cells per well) and were transfected with 3 μg TK-luc, LHRE/TK-luc, or GHREII/TK-luc reporter genes and 6 μg pCH110 (β-galactosidase expression vector, Pharmacia) per plate. 293 cells in six-well plates (0.5×10^6 cells per well) were transfected with 0.2 to 1 μg of a pcDNA3 expression vector containing the different mutant forms of GHR, 1.5 μg TK-luc, LHRE/TK-luc, or GHREII/TK-luc and 2.5 μg pCH110 per plate. In experiments using m67/TK-luc reporter gene, 2 μg Stat3 expression vector were cotransfected. Cells were incubated for 24 h with serum-free medium containing 50 nM hGH and 250 mM dexamethasone or 250 mM dexamethasone alone. Cells were lysed as described (16), and cell extracts were used for determination of luciferase and β-galactosidase activities. Luciferase activity was normalized to the β-galactosidase activity, and results are the mean (SEM) of three to five independent experiments done in triplicate.

GST-Fusion Proteins

Phosphorylated GST-fusion proteins were produced in Epicurian Coli TKB1 (Stratagene, La Jolla, CA), transformed with GST-455/620 or GST-271/620 constructs, as recommended by the manufacturer.

To dephosphorylate GST-fusion proteins, 2 μg protein were incubated for 1 h at 30 C with 10 U calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) in 20 mM HEPES, pH 7, 150 mM NaCl, 0,1% Triton X-100, and 10% glycerol.

The 293 cells were cultured in 100-mm dishes and transfected with 5 μg Stat3, Stat5, or Stat5-R618G expression vectors. Cell lysates were prepared and incubated overnight at 4 C with GST-fusion proteins bound to glutathione-Sepharose beads (2 μg per assay). After washing the beads with lysis buffer, adsorbed proteins were subjected to 7.5% SDS-PAGE and analyzed by Western blot.

The level of expression of wild type Stat5 and Stat5-R618G in 293 transfected cells was checked by immunoprecipitation of cell lysates with 1 μg anti-Stat5 antibody (Santa Cruz Biotechnology) followed by Western blot analysis.

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