GH-stimulated Ca\(^{2+}\) phosphorylation was not required for GH stimulation of transcription, indicating that any of these three tyrosines is able to independently mediate GH-induced transcription. Any of these three tyrosines are able to independently mediate GH-induced transcription, indicating redundancy in this part of the GH receptor. Tyrosine phosphorylation was not required for GH stimulation of mitogen-activated protein (MAP) kinase activity or for GH-stimulated Ca\(^{2+}\) channel activation since these pathways were normal in cells expressing a GH receptor in which all eight intracellular tyrosines were mutated to phenylalanines. Activation of Stat5 by GH was, however, abolished in cells expressing the GH receptor lacking intracellular tyrosines. This study demonstrates that specific tyrosines in the GH receptor are required for transcriptional signaling possibly by their role in the activation of transcription factor Stat5.

The binding of growth hormone (GH) to its receptor results in its dimerization followed by activation of Jak2 kinase and tyrosine phosphorylation of the GH receptor itself, as well as Jak2 and the transcription factors Stat1, -3, and -5. In order to study the role of GH receptor tyrosine phosphorylation in intracellular signaling, we constructed GH receptors in which combinations of tyrosines were mutated to phenylalanines. We identified three tyrosine residues at positions 534, 566, and 627 that were required for activation of GH-stimulated transcription of the serine protease inhibitor (Spi) 2.1 promoter. Any of these three tyrosines is able to independently mediate GH-induced transcription, indicating redundancy in this part of the GH receptor. Tyrosine phosphorylation was not required for GH stimulation of mitogen-activated protein (MAP) kinase activity or for GH-stimulated Ca\(^{2+}\) channel activation since these pathways were normal in cells expressing a GH receptor in which all eight intracellular tyrosines were mutated to phenylalanines. Activation of Stat5 by GH was, however, abolished in cells expressing the GH receptor lacking intracellular tyrosines. This study demonstrates that specific tyrosines in the GH receptor are required for transcriptional signaling possibly by their role in the activation of transcription factor Stat5.

Pituitary growth hormone (GH)\(^1\) is the major regulator of postnatal growth (1). The actions of GH at the cellular level include direct mitogenic effects (2, 3), insulin-like and insulin-antagonizing metabolic effects (4), as well as gene regulatory actions (5–7). All of these effects are initiated by the binding of GH to its receptor, which belongs to the cytokine receptor superfamily. Members of this family of receptors activate cytoplasmic tyrosine kinases of the Jak family, and these activated kinases are required for most receptor-initiated signaling pathways (8). The activated Jak2 kinase has been shown to phosphorylate several intracellular substrates including the GH receptor itself, as well as transcription factors of the Stat family (9–11).

Not only does GH activate the Jak/Stat pathway in which specific tyrosine phosphorylation of Stat1, -3, and -5 occurs in response to GH, resulting in dimerization, nuclear translocation, and binding to γ-interferon activated sequence-like elements (11–13), but we and others have previously identified several alternative signaling pathways induced by the activated GH receptor. Stimulation and tyrosine phosphorylation of MAP kinase by GH have been studied both in cultured cells (14, 15) and in vivo (16). In addition to activation of MAP kinase, its translocation to the nucleus has also been demonstrated. The activation of MAP kinase by GH is dependent upon the proline-rich box 1 domain of the GH receptor that presumably is directly involved in the binding of Jak2. When the box 1 domain is deleted or the prolines are mutated to alanines, the GH receptor is no longer able to mediate GH-induced MAP kinase activity. The functional role of MAP kinase activation in GH signaling is not known; however, it has been speculated that this activity is important for protein synthesis and possibly cell proliferation (14). The mechanism by which GH activates MAP kinase is also largely unknown, but the fact that GH can activate SHC and Grb2 indicates a mechanism similar to that utilized by receptor tyrosine kinases. We have also reported that GH is able to activate voltage-dependent Ca\(^{2+}\) channels. This activity is independent of Jak2 (17) and cannot be inhibited by tyrosine kinase inhibitors (18). The GH-induced Ca\(^{2+}\) response consists of oscillations in intracellular free Ca\(^{2+}\) concentrations of varying frequency and amplitude and has been demonstrated to be required for GH-induced transcription of the insulin gene (17). We have also shown that a region in the rat GH receptor located between residues 454 and 506 is required for the Ca\(^{2+}\)-signaling ability of the GH receptor. Finally, GH-induced tyrosine phosphorylation of cytosolic proteins has also been described, and this response has been found to be dependent on a 40-amino acid domain located between residues 476 and 516 of the porcine GH receptor (19).

Although it has been known for some time that binding of GH to its receptor leads to rapid phosphorylation of tyrosine residues in the GH receptor (9), the functional importance of GH receptor tyrosine phosphorylation remains relatively unknown. In the promyelocyte cell line FDC-P1, it has recently been shown that tyrosine phosphorylation of the GH receptor is not required for GH-stimulated proliferation (20). However, we have recently demonstrated that phosphorylation of tyrosines located close to the transmembrane region of the GH receptor is required for certain metabolic effects of GH (10, 21). For other...
members of the cytokine receptor superfamily, tyrosine phosphorylation has been implicated in signaling. In the IL-4 (22) and leukemia inhibitory factor (LIF) receptors, as well as in the signal transducer gp130 (23), phosphorylation of specific tyrosine residues is required for binding and activation of Stat factors. Furthermore, a single tyrosine located in the C-terminal domain of the prolactin receptor has been found to be crucial for transcriptional signaling (24).

While it has been possible to identify specific regions of the GH receptor as being important for various signaling pathways stimulated by GH by using deletion and substitution mutations, we now address the question of the role of GH receptor tyrosine phosphorylation in GH-mediated signal transduction. In this study, we have constructed a series of GH receptor mutants in which we have systematically substituted all intracytosolic tyrosine phosphorylation in GH-mediated signal transduction. The ability to mediate GH-stimulated transcription, activation of Stat proteins, and receptor dimerization in cells expressing these mutants was assessed (27).

GH Receptor Signaling

EXPERIMENTAL PROCEDURES

Plasmids—An Xba/I-EcoRI fragment containing the coding region of the pGH-R gene was cloned into the pMet-lg7 that contains the mouse MT-1 promoter (25). Site-directed mutagenesis of the GH receptor cDNA was performed as described (19), and mutations were confirmed by DNA sequencing. All GH-mutated GH receptor cDNAs were cloned into the pMet-lg7 expression plasmid for use in transfection studies. The plasmid pcdNAneo encoding the neo resistance gene under the transcriptional control of the cytomegalovirus promoter was from Invitrogen, San Diego, CA. The plasmid encoding β-galactosidase, pCH110, was from Pharmacia Biotech, Inc. The Spi 2.1/chloramphenicol acetyltransferase (CAT) plasmid was constructed by ligation of three copies of the Spi 2.1 GH-responsive sequence, 5'-agtATGGTCTT-GAGAAATC-3' into the plasmid TK-CAT (26) that contains the minimal thymidine kinase promoter in front of the CAT gene.

Cytosolic tyrosine phosphorylation on tyrosine kinases—CHO K1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37 °C in 5% CO₂, 95% air. Cells were grown to 80% confluence. Twenty-four h before transfection, the cells were washed once in Ham's F-12 medium without serum and starved for 24 h in serum-free medium (GC-3) containing a 1:1 mixture of minimal essential medium and Ham's F-12 medium supplemented with 10 μg of transferrin/ml (Sigma), 80 μm of insulin/ml (Novo Nordisk, Denmark), 2.5 μm glutamine, and nonessential amino acid. Just prior to transfection, the medium was changed to Opti-MEM 1 (Life Technologies, Inc.).

CHO cells were transfected using the calcium phosphate procedure with 3 μg of the β-galactosidase-encoding plasmid. 1.5 μg of the construct containing the bacterial CAT coding sequence linked to three copies of the sequence – 147 to – 103 of the Spi 2.1 promoter (1, 2), and 1.5 μg of the different mutated GH receptor plasmids. After 4 h, the cells were subjected to a glycerol shock (14% glycerol in Opti-MEM 1) for 1 min and then washed twice before fresh GC-3 medium, with or without 20 μM hGH (Novo Nordisk, Gentofte, Denmark), was added. Following 48 h of culture at 37 °C, the cells were scraped off the plate, and extracts were prepared by three consecutive freeze-thaw cycles followed by centrifugation at 15,000 × g for 10 min. GH receptor expression was analyzed in cells collected for 48 h, and all GH receptor constructs were found to be expressed at the cell surface.

The CHO cells were stably transfected with 5 μg of pGH-R cDNA using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's suggestions (75 μg of LipofectAMINE). After 8 h of exposure to the LipofectAMINE-DNA complex, the medium was changed to Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Following 24 h of additional culture, the cells were split (1:4) by trypsin treatment and cultured in 1 mg of G418/ml. After 6 days, colonies were picked and analyzed for hGH binding. Three different clones expressing the pGH-R were isolated, and three clones expressing the pGH-RmC8 were isolated and analyzed.

Receptor Binding—Binding experiments were performed using 125I-hGH (specific activity, 92 μCi/μg) and carried out for 90 min at room temperature in 25 mM Hepes (pH 7.4), 124 mM NaCl, 4 mM KCl, 2.5 mM KH₂PO₄, 1.5 mM MgCl₂, and 1 mM CaCl₂, as described previously (27).

β-Galactosidase and CAT Assay—Aliquots of the cell extracts were normalized for β-galactosidase activity and assayed for CAT activity.
The functional GH receptor (Fig. 1A) is the only cytoplasmic tyrosine in the GH receptor, can generate a functional GH receptor in CHO cells expressing wild-type or mutated GH receptors. The various mutated GH receptors are shown schematically. N denotes the amino terminus, and the shaded box represents the transmembrane domain of the GH receptor. The position of the eight intracellular tyrosines (Y) is indicated and identified by the position number. The fold induction of CAT activity by GH (20 nM) in cells cotransfected with the indicated GH receptor and the Spi 2.1/CAT reporter is shown. Results represent the average of three to eight experiments.

After 48 h in culture with or without 20 nM GH, the functional activity of the transfected GH receptor was analyzed by its ability to direct GH-induced expression of CAT activity. In the series of truncated GH receptors shown in Fig. 1A, it was found that the 80 most C-terminal amino acids could be deleted without loss of GH induction, but when 122 amino acids were deleted (GH 1–516), no GH stimulation of CAT activity was observed. The mutation of individual tyrosines to phenylalanines in the intracellular domain of the GH receptor did not affect the signaling ability of the GH receptor since all eight receptors were able to mediate GH-stimulated CAT activity (Fig. 1B). Similar results were obtained using the rat GH receptor in which the intracellular tyrosines were individually mutated to phenylalanines. In contrast, when tyrosine residues were mutated to phenylalanines in combination and sequentially from the C terminus, signaling by the GH receptor was lost when the four most C-terminal tyrosines were mutated to phenylalanines (Fig. 1C). These results indicate a role for two or more of these tyrosines in signaling. Finally, we constructed mutated GH receptors in which seven out of the eight cytoplasmic tyrosines were mutated to phenylalanines. The results from this series of GH receptor mutants revealed that any one of three tyrosines, 534, 566, or 627, when present, will affect the signaling ability of the GH receptor since all eight receptors were able to mediate GH-induced CAT activity (Fig. 1D).

The role of GH receptor tyrosine phosphorylation in GH-induced MAP kinase activation was evaluated in CHO cells stably transfected with either the wild-type or the GH receptor mC8 mutant lacking all intracellular tyrosines. The number of GH receptors in the two clones shown in Fig. 3 was comparable, expressing approximately 40,000 receptors/cell. GH-induced MAP kinase activity was the same in cells expressing either the wild-type or the mC8 mutant GH receptor, but no MAP kinase activation was seen in untransfected CHO cells (Fig. 3). These results demonstrate that tyrosine phosphorylation of the GH receptor is not required for GH-induced activation of MAP kinase activity.

The domain of the GH receptor required for GH-induced oscillations in intracellular free Ca²⁺ concentrations has been found to overlap with the domain required for transcriptional signaling, suggesting a role for Ca²⁺ in GH-induced transcriptional signaling. We have, therefore, examined the ability of the wild-type and the mC8 mutant GH receptors to mediate GH-induced Ca²⁺ oscillations by fluorescence image microscopy of Fura-2-loaded CHO cells. Untransfected CHO cells (Fig. 4A), dexamethasone-refractory cells expressing the wild-type (Fig. 4B), or the mC8 mutant (Fig. 4C) GH receptor exhibited GH-induced Ca²⁺ oscillations similar to those described previously. Similarly, pools of transfected CHO cells expressing wild-type (Fig. 4D) or mC8 mutant (Fig. 4E) GH receptors showed the typical GH-induced Ca²⁺ oscillations of varying frequency and amplitude. The Ca²⁺ response was observed in the majority of GH receptor-expressing cells analyzed but was never seen in untransfected CHO cells.

Since cytokine receptor phosphorylation has been implicated in Stat factor activation for the IL-4 and LIF receptors, we investigated the role of GH receptor phosphorylation in the activation of Stat5 by gel retardation assay. Nuclear extracts from control and GH-stimulated cells expressing wild-type or mC8 mutant GH receptors were isolated. Using the GH response element from the Spi 2.1 promoter as a probe, a GH-induced band was observed using nuclear extracts from cells expressing the wild-type GH receptor (Fig. 5A, lane 4). In contrast, no specific bands were observed using nuclear extracts from cells expressing the mC8 mutant GH receptor (Fig. 5A, lanes 5 and 6). The GH-induced band was specific since it could be inhibited by unlabeled oligonucleotide but not by an unrelated oligonucleotide (Fig. 5C, lanes 15 and 16). The presence of Stat5 in the observed complexes was tested by using Stat5 antibodies in gel retardation assays. A supershift of the GH-induced bands was observed when the Stat5 antibody was present in the binding reaction, whereas a preimmune serum did not affect the formation of the GH-induced complexes (Fig. 5C, lanes 17 and 18). Antibodies against Stat1 or Stat3 did not cause a supershift of the GH-induced bands. The quality of the

Fig. 1. Growth hormone-induced CAT activity in CHO cells expressing wild-type and mutated GH receptors. The various mutated GH receptors are shown schematically. N denotes the amino terminus, and the shaded box represents the transmembrane domain of the GH receptor. The position of the eight intracellular tyrosines (Y) is indicated and identified by the position number. The fold induction of CAT activity by GH (20 nM) in cells cotransfected with the indicated GH receptor and the Spi 2.1/CAT reporter is shown. Results represent the average of three to eight experiments.

Fig. 2. Amino acid sequence of the porcine GH receptor domains containing the three tyrosine residues found to be involved in transcriptional signaling. The number above the tyrosine refers to the position of the tyrosine residue in the porcine GH receptor precursor.

Fig. 3. MAP kinase activity in GH-stimulated CHO cells expressing wild-type or mC8 mutant GH receptors. MAP kinase activity was measured as described under "Experimental Procedures," and cells were stimulated for 0, 5, or 10 min with 20 nM GH. Results shown are from one representative experiment.
nuclear extracts was tested by DNA binding activity to the cyclic AMP response element from the α-chorionic gonadotropin promoter. All extracts were found to contain proteins capable of binding to this promoter element (Fig. 5B).

**DISCUSSION**

We have demonstrated in this study that three tyrosine residues in the intracellular domain of the GH receptor are involved in transcriptional signaling. Specific tyrosine phosphorylation of GH receptors in response to GH has been previously observed in several different cell types (9, 29, 30), and even highly purified GH receptor preparations were found to exhibit tyrosine kinase activity phosphorylating the GH receptor (9). However, it was recently shown that the GH receptor-associated tyrosine kinase was Jak2 (31) and that binding and activation of Jak2 by GH was dependent upon a proline-rich domain in the GH receptor (15). The proline-rich domain is required for receptor signaling since deletion of this domain results in a receptor that is unable to activate signal transduction (17, 32, 33). We and others have previously identified the C-terminal 184 amino acids of the rat GH receptor as being required for GH-stimulated transcription of the Spi 2.1 (32, 33) and insulin (34) genes. However, the signaling properties of this domain are not known, although it has been implicated in GH-stimulated Ca\(^{2+}\) channel activation (17). By way of cotransfection of truncated porcine GH receptor cDNA with an Spi 2.1 promoter/CAT construct, we found that GH stimulation of CAT activity can be abolished when 122 amino acids were deleted from the C terminus of the porcine GH receptor. In order to examine the possible effect of GH receptor tyrosine phosphorylation on transcriptional signaling, we substituted individual tyrosine residues with phenylalanines. All GH receptors containing single mutations were able to mediate GH-induced CAT activity. The variation in GH induction observed (Fig. 1B) does not reflect a true variation in signaling ability, but it most likely reflects differences in expression levels of the transfected GH receptors caused by differences in plasmid purity. When different preparations of the wild-type GH receptor encoding plasmid were used in the transcription assay, GH induction varied from 4- to 12-fold.

Mutation of multiple tyrosines to phenylalanines as shown in Fig. 1, C and D, revealed that three tyrosine residues to phenylalanines as shown in Fig. 1, C and D, revealed that three tyrosine residues at positions 534, 566, and 627 are involved in signaling and that each of these tyrosines can function independently. A similar redundancy has been observed in receptors for IL-4 (22), LIF, and the signal transducer gp130 (23). Three tyrosines in the intracellular domain of the LIF receptor and four tyrosines in gp130 were found to be involved in Stat3 activation. Furthermore, in the IL-4 receptor, two tyrosines have been implicated in the activation of Stat6. In all cases, it has been implicated that a direct interaction between the SH2 domain of the Stat factor and the phosphorylated tyrosine on the receptor is a prerequisite for Stat activation. The specificity of SH2 domain interaction with phosphorylated tyrosines appears to reside within the three amino acids C-terminal to the phosphorylated tyrosine (35). In the LIF receptor and gp130, all five tyrosines found to be involved in Stat3 activation have con-
served amino acids in the +1 to +3 positions. Similarly, in the IL-4 receptor, the identical amino acids are present in the +1 and +3 positions relative to the tyrosines involved in Stat6 activation. In the prolactin receptor that is 35% identical to the GH receptor, a single tyrosine residue has been identified as being responsible for activation of β-casein gene transcription.

Interestingly, this tyrosine is conserved in prolactin and GH receptors of all species examined so far. Considering that both prolactin and GH stimulate Stat5 factors and that Stat5 has been shown to bind to hormone-responsive elements in the β-casein (36, 37) and Spi 2.1 promoters, it seems likely that this C-terminal tyrosine is directly involved in Stat5 activation. In contrast to the LIF and IL-4 receptors and gp130 in which the tyrosines involved in Stat activation have very similar +1 to +3 positions, the three tyrosines identified in this study have varying C-terminal flanking amino acids. Except for the threonine residue at the +3 position relative to tyrosines 627 and 566, no other identities or similarities exist among these positions. The C-terminal flanking amino acids are conserved in all species of cloned GH receptors until now. This raises the possibility that the three tyrosines use different signaling pathways for mediating GH-stimulated Spi 2.1 transcription by binding separate signaling molecules having different SH2 domains. Another explanation could be that all three tyrosines are involved in Stat5 activation, but each tyrosine activates only one of the Stat5 isoforms, A or B (38, 39).

In CHO cells expressing the GH receptor mutant lacking all intracellular tyrosines (mC8), no activation of Stat5 could be observed, whereas in cells expressing the wild-type GH receptor GH was able to induce binding of Stat5 to the Spi 2.1 promoter. In contrast it was previously shown that in FDC-P1 promyeloid cells expressing wild-type or tyrosine-deficient GH receptors intracellular tyrosines are not required for GH activation of Stat1 or -3 binding to the sis-inducible element from the c-fos promoter (20). In the same study, GH-induced Jak2 activation in FDC-P1 cells was not affected by the lack of intracellular tyrosines in the GH receptor. We also observed that GH can induce Jak2 activation in CHO cells expressing the tyrosine-deficient GH receptor mutant as well as GH-stimulated MAP kinase activity. GH-induced oscillations in [Ca2+]i were similarly unaffected by the mutation of all intracellular tyrosines to phenylalanines. This is in agreement with our previous observation that Jak2 kinase activity is not required for GH-stimulated Ca2+ signaling and that tyrosine kinase inhibitors are not able to block the GH-induced rise in [Ca2+]i (17, 18).

In conclusion we have shown that three tyrosines in the C-terminal domain of the GH receptor function as mediators of GH-induced transcription of the Spi 2.1 gene. These tyrosines are, however, not required for GH-induced MAP kinase activity nor for GH-induced Ca2+ signaling. The transcriptional signaling pathway presumably involves Stat5 activation by specific binding of Stat5 to the phosphorylated tyrosines followed by phosphorylation of Stat5 that leads to dimerization and DNA binding. Whether Stat5 binds directly to the phosphorylated tyrosines in the GH receptor or via adapter proteins is not known at present. The observation that tyrosine-deficient GH receptors can still mediate GH effects on proliferation and Stat1 and -3 activation in FDC-P1 cells (20) suggests that GH activates different Stat proteins by distinct signaling pathways. The activation of Stat1 and -3 may be mediated by phosphorylated tyrosines on Jak2 itself, whereas Stat5 activation requires phosphotyrosines in the C-terminal domain of the GH receptor. This would imply that the proliferative effects of GH are mediated at least in part by Stat1 and -3 activation, whereas the effects of GH on cell-specific gene transcription require Stat5.

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