Growth Hormone-dependent Phosphorylation of Tyrosine 333 and/or 338 of the Growth Hormone Receptor*

Joyce A. VanderKuur‡§, Xueyan Wang‡, Liyin Zhang‡, Giovanna Allevato‡, Nils Billestrup‡, and Christin Carter-Sul‡**

From the ‡Department of Physiology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0622 and the §Hagedorn Research Laboratory, Nils Stensrøeje 6, DK-2820 Gentofte, Denmark

Many signaling pathways initiated by ligands that activate receptor tyrosine kinases have been shown to involve the binding of SH2 domain-containing proteins to specific phosphorylated tyrosines in the receptor. Although the receptor for growth hormone (GH) does not contain intrinsic tyrosine kinase activity, GH has recently been shown to promote the association of its receptor with a tyrosine kinase, to activate it, and to promote the tyrosyl phosphorylation of both GH receptor (GHR) and JAK2. In this work, we examined whether tyrosines 333 and/or 338 in GHR are phosphorylated by JAK2 in response to GH. Tyrosines 333 and 338 in rat full-length (GHR1, 454) and truncated (GHR1, 441) receptor were replaced with phenylalanines and the mutated GHRs expressed in Chinese hamster ovary cells. These substitutions caused a loss of GH-dependent tyrosyl phosphorylation of truncated receptor and a reduction of GH-dependent phosphorylation of the full-length receptor. Consistent with Tyr333 and/or Tyr338 serving as substrates for JAK2, these substitutions resulted in a loss of tyrosyl phosphorylation of truncated receptor in an in vitro kinase assay using substantially purified GH-GHR-JAK2 complexes. The Tyr to Phe substitutions did not substantially alter GH-dependent JAK2 association with GHR or tyrosyl phosphorylation of JAK2. These results suggest that Tyr333 and/or Tyr338 in GHR are phosphorylated in response to GH and may therefore serve as binding sites for SH2 domain-containing proteins in GH signal transduction pathways.

Ligand binding to membrane receptors with intrinsic tyrosine kinase activity has been shown to result in the phosphorylation of multiple tyrosines in the receptors themselves (reviewed in Ref. 1). Once phosphorylated, these tyrosines have been shown to bind the Src homology 2 (SH2) domain of signaling molecules that mediate responses to GH. Since the receptor for growth hormone (GH) does not contain intrinsic tyrosine kinase activity, GH has recently been shown to promote the association of its receptor with a tyrosine kinase, to activate it, and to promote the tyrosyl phosphorylation of both GH receptor (GHR) and JAK2. In this work, we examined whether tyrosines 333 and/or 338 in GHR are phosphorylated by JAK2 in response to GH. Tyrosines 333 and 338 in rat full-length (GHR1, 454) and truncated (GHR1, 441) receptor were replaced with phenylalanines and the mutated GHRs expressed in Chinese hamster ovary cells. These substitutions caused a loss of GH-dependent tyrosyl phosphorylation of truncated receptor and a reduction of GH-dependent phosphorylation of the full-length receptor. Consistent with Tyr333 and/or Tyr338 serving as substrates for JAK2, these substitutions resulted in a loss of tyrosyl phosphorylation of truncated receptor in an in vitro kinase assay using substantially purified GH-GHR-JAK2 complexes. The Tyr to Phe substitutions did not substantially alter GH-dependent JAK2 association with GHR or tyrosyl phosphorylation of JAK2. These results suggest that Tyr333 and/or Tyr338 in GHR are phosphorylated in response to GH and may therefore serve as binding sites for SH2 domain-containing proteins in GH signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Recombinant DNA-derived 22,000-dalton hGH was a gift of Lilly. Recombinant protein A-agarose was from Repligen, and the protein assay (BCA) was from Pierce. Triton X-100, aprotinin, and leupeptin were purchased from Boehringer Mannheim. Molecular weight standards (unstained) and ovalbumin were purchased from Sigma, prestained molecular weight standards were from Life Technologies Corporation.

*This work was supported by Research Grants DK34171 and DK48283 from the National Institutes of Health (to C. C. -S.). Computer studies were supported in part by the General Clinical Research Center at the University of Michigan, funded by Grant M01 RR00042 from the National Center for Research Resources, National Institutes of Health, United States Public Health Service. The costs of publication of this paper must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§Recipient of Postdoctoral Fellowships from the National Institutes of Health (ST32-DK67245) and the Arthritis Foundation.

†Recipient of a Rachivalpulla dissertation Grant from the University of Michigan.

**To whom correspondence should be addressed: Dept. of Physiology, The University of Michigan Medical School, Ann Arbor, MI 48109-0622. Fax: 313-936-8813.

1 The abbreviations used are: GHR, growth hormone receptor; GH, growth hormone; hGH, human growth hormone; rGHR, rat growth hormone receptor; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; KRP, Krebs-Ringer phosphate buffer.
Receptor numbers were compared by incubating cell monolayers with affinities of the different GHR appeared not to differ (data not shown), incubated for the indicated times with hGH at 37°C in 95% air, 5% otherwise.

Receptors at positions 333 and 338 (GHR1–638Y333F,Y338F) were compared by immunoprecipitation and Western blotting—Confluent CHO cells were incubated in the absence of serum overnight (32). Cells were incubated with labeled GH at 37°C in the presence of 2 μM leupeptin. Immunoprecipitates were subjected to SDS-PAGE (with the amount of sample normalized to protein) followed by Western blot analysis with the indicated antibody using the enhanced chemiluminescence detection system (33). In some experiments, the blots were rinsed and reprobed with a second antibody.

Formation of Cross-linked 125I-hGH Receptor Complexes and GH Binding Assay—Human GH was labeled with 125I to an estimated specific activity of ∼ 90 μCi/μg using chloramine T by the University of Michigan Reproductive Sciences Training Grant Core Facility. As described previously (34), cells were washed twice with serum-free medium overnight and then washed with Krebs-Ringer phosphate buffer (KRP) containing 1% bovine serum albumin. 125I-GH (12 × 10^6 counts/min/100-mm dish, 8-40 ng/ml) in KRP, 1% bovine serum albumin was added to the cells in the presence or absence of 1 μM unlabeled hGH and incubated for 1 h at 25°C. After extensive washing with KRP, 1% bovine serum albumin (100 μg/ml; 10 μg/ml aprotinin, 10 μg/ml leupeptin) and resuspended in 200 μl of buffer containing 250 μg/ml aprotinin and 250 μg/ml leupeptin. In vitro phosphorylation was carried out by adding 20 μl of 50 μM hGH, 1% Triton X-100, 7.6 μM, vehicle containing unlabeled ATP (5 μM), or unlabeled ATP (5 μM) plus (γ-32P)ATP (200 μCi) and incubating for 10 min at 30°C as described previously (35, 36). The reaction was stopped by the addition of 1 ml of ice-cold NHT buffer containing 10 mM EDTA, pH 7.6, followed by extensive washing. Immunoprecipitated proteins were eluted from the immunomatrixes by boiling in 200 μl of 150 μM Tris, pH 6.8, 3% SDS, 3% β-mercaptoethanol, 30% glycerol, and 0.03 mM bromphenol blue and analyzed by SDS-PAGE followed by autoradiography or Western blotting using αPY as described above.

SDS-PAGE and Densitometry—SDS-PAGE was performed using 3-10% gradient gels (30.5, 45.0, and 60.0 μM leucylamidobisacylamide) as described previously (36). SDS-PAGE gels described in Figs. 2, 6, and 7 contained prestained molecular weight standards: ovalbumin (43,600), bovine serum albumin (70,800), phosphorylase b (105,000), and myosin (203,000). SDS-PAGE gels described previously (28). Because the affinities of the different GHR appeared not to differ (data not shown), receptor numbers were compared by incubating cell monolayers with 125I-hGH (1-6 ng/ml) for 1 h at 25°C. Cells were washed with ice-cold KRP and lysed with 1 n NaOH. Radioactivity associated with the cells was determined by counting cell lysates in a γ counter. Results were normalized to protein content and corrected for nonspecific binding, which was determined by incubating cell monolayers with 32P-hGH in the presence of 2 μM unlabeled hGH. The relative abilities of the different GH receptors to bind 125I-hGH were similar when binding was determined by incubating the cells with 125I-hGH for 1 h at 25°C or overnight at 4°C.

In Vivo Kinase Assay—Cells were grown to confluence and deprived of serum overnight as described previously (35). Cells were then incubated in the absence or presence of 100 ng/ml (4.5 μM) GH at 25°C for 1 h. (Fig. 3) cells were washed with ice-cold 125I-hGH at 37°C for 15 min (Fig. 4) and lysed in HVTDP buffer. Cell lysates were centrifuged at 230,000 × g for 1 h. Supernatants were then incubated with αGH (1,100 ng/ml) for 2 h at 8°C. Immunocomplexes were precipitated with immobilized protein A, and immunomatrixes were extensively washed with NHT (50 μM HEPES, 50 μM NaCl, 50 μM EDTA, pH 7.6) and resuspended in 200 μl of buffer C containing 250 μg/ml aprotinin and 250 μg/ml leupeptin. In vitro phosphorylation was carried out by adding 20 μl of 50 μM hGH, 1% Triton X-100, 7.6 μM, vehicle containing unlabeled ATP (5 μM), or unlabeled ATP (5 μM) plus (γ-32P)ATP (200 μCi) and incubating for 10 min at 30°C as described previously (35, 36). The reaction was stopped by the addition of 1 ml of ice-cold NHT buffer containing 10 mM EDTA, pH 7.6, followed by extensive washing. Immunoprecipitated proteins were eluted from the immunomatrixes by boiling in 200 μl of 150 μM Tris, pH 6.8, 3% SDS, 3% β-mercaptoethanol, 30% glycerol, and 0.03 mM bromphenol blue and analyzed by SDS-PAGE followed by autoradiography or Western blotting using αPY as described above.

RESULTS

Ability of GH to Promote Tyrosyl Phosphorylation of GHR Lacking Tyr333 and Tyr338—Previous results indicated that GHR1–454 is tyrosyl-phosphorylated in response to GH (7), indicating that 1 or more of the 4 tyrosines present in the cytoplasmic domain of this truncated receptor is a substrate of the GH-activated, GHR-associated JAK2 tyrosine kinase. To determine which of the 4 tyrosines present in this truncated receptor is phosphorylated in response to GH, we replaced Tyr333 and Tyr338 in both wild-type (GHR1–638) and GHR1–454 with phenylalanines (Fig. 1), expressed the mutated receptors in CHO cells, compared the relative levels of 125I-hGH binding in the different cell lines, and examined whether GH stimulates tyrosyl phosphorylation of these mutated receptors. 125I-hGH binding to cells expressing GHR1–638Y333F,Y338F, GHR1–454 and GHR1–454Y333F,Y338F was 88 ± 2% (n = 2), 71 ± 4% (n = 12), 41 ± 5% (n = 10), respectively, that of cells expressing wild-type GHR1–638 (Fig. 1). GH-GHR1JAK2 complexes were prepared from GH-treated cells using αGH. Because GH is not phosphorylated in the absence of GH (6, 7), the amount of tyrosyl phosphorylated GHR observed in the αGH precipitates from GH-treated cells reflects the amount of GH-dependent phosphorylation. When GH-GHR1JAK2 complexes are precipitated from CHO cells expressing truncated receptor and Western-blotted with αPY, two tyrosyl phosphorylated proteins are detectable (Fig. 2, lane F) as reported previously (7). The lower, 2 T. King and C. Carter-Su, unpublished observation.
Phosphorylation of Tyr\(^{333}\) and/or Tyr\(^{338}\) of GHR

**Fig. 1.** Wild-type and mutated GHRs expressed in CHO cells. Denoted are the extracellular domain, the transmembrane (hatched area), and the cytoplasmic domain of the mutated rat liver GHR. Tyrosyl residues in the cytoplasmic domain are denoted by Y. Phenylalanine residues that were substituted for tyrosyl residues are denoted by F. Binding data were determined as described under “Experimental Procedures.”

When GH-GHR-JAK2 complexes are precipitated using \(\alpha\)GH from GH-treated CHO cells expressing wild-type receptor and Western-blotted with \(\alpha\)PY, a broad band migrating with M, \(\sim 120,000-130,000\) is observed (Fig. 2, lane B), as reported previously (7). Western blotting with \(\alpha\)GH and \(\alpha\)JAK2 indicates that this band contains both GHR and JAK2, with JAK2 migrating as a rather narrow band (M, \(\sim 130,000\)) (see Fig. 6) and GHR migrating as a diffuse band (M, \(\sim 120,000\)) with and just below JAK2 (6, 7). In \(\alpha\)PY blots of \(\alpha\)GH precipitates from GH-treated CHO cells expressing Y333F Y338F full-length receptor, a diffuse band migrating with a M, appropriate for both GHR and JAK2, is also observed (Fig. 2, lane D). The diffuse nature of the band indicates that the mutated receptor is phosphorylated, suggesting that tyrosines other than 333 and/or 338 in GHR are phosphorylated in response to GH. However, the significantly reduced intensity of this band (by 76 \pm 5\%, n = 3) compared with that obtained with wild-type receptor suggests that phosphorylation of Tyr\(^{333}\) and/or Tyr\(^{338}\) contributes to the level of GHR phosphorylation observed in wild-type receptor.

Ability of Substantially Purified GH-GHR Complexes to Incorporate Phosphate in an In Vitro Kinase Assay—To examine whether Tyr\(^{333}\) and/or Tyr\(^{338}\) are likely to be phosphorylated by the GHR-associated JAK2 kinase, GH-GHR-JAK2 complexes were substantially purified from GH-treated CHO cells using \(\alpha\)GH and incubated with \(\gamma\)\(^{32}\)P\(\)ATP. Fig. 3a illustrates that \(\gamma\)\(^{32}\)P is incorporated into proteins migrating with molecular
weights appropriate for both GHR and JAK2 when GH-GHR:AK2 complexes are prepared from CHO cells expressing GHR1–638 (Fig. 3a, lane B), as reported previously (7). When a similar experiment is performed using the Y333F,Y338F mutated receptor, 32P is incorporated almost exclusively into a band corresponding in size to JAK2 (Fig. 3a, lane D). The amount of mutated receptor is assumed to be less (by 40%) than the amount of unmutated receptor based upon 125I-hGH binding data (Fig. 1). To ensure that a 40% reduction in the number of GHR could not account for the inability to detect phosphorylated Tyr → Phe truncated receptor, lanes C and D were exposed to film for a longer period of time sufficient to almost triple the phosphorylation signal for GHR1–454. Even with the longer exposure, no band co-migrating with phosphorylated truncated receptor was observed (Fig. 3a, lane F). A faint band of unknown origin may be seen migrating slightly ahead of where truncated GHR would be (Fig. 3a, lane F).

To confirm that in the in vivo kinase assay, there is a difference between mutated and unmutated receptor in the amount of phosphate incorporated into tyrosyl, as opposed to seryl and threonyl, residues, GH-GHR+AK2 complexes were prepared from CHO cells treated with 100 ng/ml (4.5 nM) GH for 15 min at 37 °C and incubated in the absence and presence of unlabeled ATP at the same concentration of ATP (5 μM) used in the [γ-32P]ATP experiment. Kinase assay-dependent changes in the amount of tyrosyl phosphorylation of GHR were assessed by Western blotting with αPY. An ATP-dependent tyrosyl phosphorylation of a protein migrating with apparent molecular weight appropriate for both GHR and JAK2, was observed when GHR was prepared from CHO cells expressing truncated receptor (Fig. 3b, compare lanes B and C), but not when it was prepared from cells expressing truncated receptor with the Tyr to Phe substitution (Fig. 3b, compare lanes E and F). As in Fig. 3a, the intensity of the JAK2 band from mutated versus unmutated cells was reduced approximately to the same extent as binding of 125I-hGH (Fig. 1). Even when lanes E and F were exposed to film for a substantially longer period of time (Fig. 3b, lanes H and I) to compensate for the 40% decrease in GH binding in the cells expressing mutated receptor and making the JAK2 signal comparable for the mutated and unmutated receptors, no band corresponding to the mutated receptor was detectable.

Size of GHR Lacking Tyr333 and Tyr338—The experiments described for Figs. 2 and 3 provide evidence that the amount of phosphate incorporated into GHR lacking Tyr333 and Tyr338 both in vivo and in the in vitro kinase assay is reduced compared with unmutated GHR by more than can be accounted for by differences in the amount of GHR expressed in the corresponding cell lines. This is consistent with GH-promoting the tyrosyl phosphorylation of Tyr333 and/or Tyr338 and with one or both of these tyrosines serving as a substrate of a GHR-associated kinase, presumably JAK2. However, the reduced level of phosphorylation observed for GHR lacking Tyr333 and Tyr338 could also potentially arise if in comparison to their nonmutated counterparts, the mutated receptors: 1) were more susceptible to proteolysis so that they lacked tyrosines other than 333 and 338 that are sites of phosphorylation or 2) had a substantially reduced ability to associate with or to activate JAK2.

To verify that mutation of Tyr333 and Tyr338 to Phe did not result in adventitious proteolysis of full-length and truncated receptors to the extent that potential alternative phosphorylation sites were deleted, CHO cells expressing the various GHRs were incubated with 125I-hGH, followed by the cross-linking reagent disuccinimidyl suberate. Fig. 4 illustrates that when the molecular weight of hGH (22,000, Ref. 37) is taken into account, the various cross-linked 125I-hGH-GHR complexes migrate as proteins of the appropriate size (M, ~134,000 for full-length, M, ~95,000 for truncated receptor). In Fig. 4, a significantly greater portion of the 125I-hGH-GHR1–638,Y333F,Y338F complexes compared with other 125I-hGH-GHR complexes appeared to be degraded, migrating as if the receptor was truncated at amino acid ~415. However, this large difference was not reproducible. In the three cross-linking experiments performed, the amount of 125I-hGH cross-linked to full-length mutated GHR was only 26 ± 37% less than the amount of 125I-hGH cross-linked to full-length GHR. This suggests that the reduced (by ~80%) phosphorylation observed for GHR1–638,Y333F,Y338F-JAK2 complexes compared with GHR1–638-JAK2 complexes cannot be attributed to a comparable reduction in the amount of intact receptor. Despite the presence of substantial amounts of degraded 125I-hGH-GHR1–638 complexes corresponding in size to GHR1–415 in Fig. 5, one does not see in Fig. 2 a phosphorylated band corresponding in size to this truncated receptor. This provides ad-

---

3 In contrast to Fig. 2, in vivo tyrosyl phosphorylation of truncated receptor is not evident in Fig. 3b (i.e. no 80-kDa band is detected in lane B). This apparent discrepancy results from the difference in GH concentrations used in the two experiments. In Fig. 2, cells were incubated with a maximally stimulatory concentration of GH (500 ng/ml, 22 nm) to maximize the amount of GHR phosphorylation. For Fig. 3b, cells were incubated with a submaximal concentration of GH (100 ng/ml, 4.5 nm) to enable additional phosphorylation of these proteins to occur in the in vitro kinase assay. The result was the same whether the incubation with GH was for 15 min for 37 °C as in Fig. 3b or for 1 h at 25 °C as for Fig. 3a (data not shown).
Although the amount of JAK2 associated with receptor, and to compare the ability of the mutated and non-mutated \((GHR)\) as noted were incubated for 5 min at 37°C without \((CHO)\) cells expressing various tyrosyl phosphorylation of JAK2. In addition, evidence of the ability of JAK2 to associate with these mutant receptors. To provide more direct phosphorylation of JAK2 with GHR and JAK2 Activation—

**Fig. 6.** The ability of mutated GHR to elicit GH-dependent tyrosyl phosphorylation of JAK2. CHO cells expressing various GHR as noted were incubated for 5 min at 37°C without \((GHR)\) and Western-blotted with \(\alpha PY\). The migration of JAK2 is indicated. Lanes A–H are directly comparable with lanes A–H in Fig. 2, since samples were prepared using aliquots from the same cell lines were separated on the same gel and Western-blotted together.

**Fig. 7.** The ability of GH to stimulate tyrosyl phosphorylation of cellular proteins in CHO cells expressing mutated GHR. CHO cells expressing various GHR as noted were incubated at 37°C with 23 nm (500 ng/ml) hGH for the times indicated and then lysed with boiling SDS sample buffer diluted (20:80) with lysis buffer. Proteins were separated by SDS-PAGE and Western-blotted with \(\alpha PY\). The migration of prestained molecular weight standards \((\times 10^3)\) are indicated between lanes F and G. The migration of p121, p97, p42, and p39 is indicated by arrows on the left.

Phosphorylation of Tyr\(^{333}\) and/or Tyr\(^{338}\) of GHR

Additional evidence that Tyr\(^{301}\) (predicted to be the only tyrosine present in Tyr \(\rightarrow\) Phe mutated GHR\(_{1-415}\)) is not phosphorylated in response to GH, supporting our overall conclusion that Tyr\(^{333}\) and Tyr\(^{338}\) but not Tyr\(^{301}\) \((GHR)\) are phosphorylated in response to GH.

**Ability of GHR Mutants to Mediate GH-promoted Association of JAK2 with GHR and JAK2 Activation—**

The ability of mutated GHR to elicit GH-dependent tyrosyl phosphorylation of JAK2, CHO cells expressing various GHR as noted were incubated for 5 min at 37°C without \((CHO)\) cells expressing various tyrosyl phosphorylation of JAK2. In addition, evidence of the ability of JAK2 to associate with these mutant receptors. To provide more direct phosphorylation of JAK2 with GHR and JAK2 Activation—

**Ability of GHR Mutants to Mediate GH-promoted Association of JAK2 with GHR and JAK2 Activation—**

The results of Figs. 2 and 3 showing the presence of a tyrosyl-phosphorylated protein migrating with a molecular weight appropriate for JAK2 in aGH immunoprecipitates from GH-treated cells indicate that JAK2 co-precipitates with the mutated full-length and truncated GHR. This suggests that JAK2 is capable of associating with these mutant receptors. To provide more direct evidence of the ability of JAK2 to associate with these mutant receptors, and to compare the ability of the mutated and non-mutated receptors to bind JAK2, GH-GHR-JAK2 complexes were immunoprecipitated using \(\alpha GH\) and Western-blotted with \(\alpha JAK2\) (Fig. 5). JAK2 was found to associate with both the nonmutated \((lanes C and E)\) and mutated \((lanes A and G)\) receptors. Although the amount of JAK2 associated with GHR\(_{1-454}\)Y333F, Y338F compared with GHR\(_{1-454}\) in Fig. 5 appeared to be substantially reduced, this finding was not reproducible. In three experiments, the amount of JAK2 associated with GHR\(_{1-454}\)Y333F, Y338F was 115 ± 31% the amount associated with GHR\(_{1-454}\). The amount associated with mutated, full-length GHR was 67 ± 1% the amount associated with wild-type GHR. Thus, JAK2 association with GHR does not depend upon Tyr\(^{333}\) and/or Tyr\(^{338}\). Furthermore, the substantial reduction in the amount of phosphorylated receptor observed when Tyr\(^{333}\) and Tyr\(^{338}\) are mutated to Phe cannot be attributed to a comparable decreased level of JAK2 associated with these GHR.

![Phosphorylation of Tyr\(^{333}\) and/or Tyr\(^{338}\) of GHR](image)

To determine whether Tyr\(^{333}\) and/or Tyr\(^{338}\) are required for activation of JAK2, we compared the abilities of the mutated and unmutated GHR to mediate GH-dependent tyrosyl phosphorylation of JAK2. Tyrosyl phosphorylation of JAK kinases is thought to be due to autophosphorylation and thus to reflect JAK activation (6, 8), a hypothesis supported by the finding that tyrosyl phosphorylation of JAK2 correlates well with JAK2 activation by GH.\(^4\) JAK2 was precipitated from CHO cells expressing the various GHRs using \(\alpha JAK2\) and tyrosyl phosphorylation of JAK2 was assessed by Western blotting with \(\alpha PY\). Fig. 6 illustrates that for CHO cells expressing the

\(^4\) E. Adkins, G. Campbell, and C. Carter-Su, unpublished observation.
The results presented in this work provide strong evidence that Tyr<sub>333</sub> and Tyr<sub>338</sub> do not seem to be required for GH association with JAK2 tyrosine kinase that phosphorylates these tyrosine(s), since receptors lacking these tyrosines are phosphorylated to a significantly reduced extent (full-length) or not at all (truncated receptor) compared with the same sized receptors containing these tyrosines. Presumably, this is the GH-associated JAK2 tyrosine kinase that phosphorylates these tyrosine(s), since the truncated receptor is phosphorylated when GH-GHR<sub>1–454</sub> complexes are precipitated with αGH and incubated with [γ<sup>32</sup>P]ATP, whereas the truncated receptor lacking Tyr<sub>333</sub> and Tyr<sub>338</sub> is not. Although Tyr<sub>333</sub> seems the most likely candidate based upon sequence analysis, additional studies will be required to determine which of the two tyrosines (333 or 338) is phosphorylated. Whether or not these are the only tyrosines in GHR<sub>1–454</sub> that are phosphorylated is not known, since it is possible that phosphorylation of Tyr<sub>333</sub> and/or Tyr<sub>338</sub> is required for the subsequent phosphorylation of Tyr<sub>391</sub> and Tyr<sub>437</sub>. Although it seems unlikely given the conservative nature of the amino acid substitution, our results cannot rule out the alternative possibility that Tyr<sub>333</sub> and Tyr<sub>338</sub> are not themselves phosphorylated but rather, mutating Tyr<sub>333</sub> and Tyr<sub>338</sub> to Phe alters the ability of Tyr<sub>391</sub> and/or Tyr<sub>437</sub> to be phosphorylated.

Tyr<sub>333</sub> and/or Tyr<sub>338</sub> appear not to be the only tyrosines phosphorylated in response to GH, since GH appears to stimulate the tyrosyl phosphorylation of the full-length, Tyr<sub>333</sub> and Tyr<sub>338</sub> mutated receptor. The 6 tyrosines between amino acids 454–638 are the most likely candidates because tyrosines other than 333 and/or 338 present in GHR<sub>1–454</sub> appear not to be phosphorylated to any great extent. Multiple phosphorylated tyrosines in GHR would be consistent with multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.

The finding that Tyr<sub>333</sub> and/or Tyr<sub>338</sub> are likely to be phosphorylated in response to GH raises the question of whether either or both serve as binding sites for specific SH2 domains. Neither tyrosine, with its surrounding amino acids, closely resembles a high affinity binding site (as currently defined) of various SH2 domains (reviewed in Ref. 1). Multiple phosphorylated tyrosines in GHR<sub>1–454</sub> appear not to be phosphorylated to any great extent. Multiple phosphorylated tyrosines in GHR would be consistent with multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.

The results presented in this work provide strong evidence that Tyr<sub>333</sub> and Tyr<sub>338</sub> do not seem to be required for GH association with JAK2 tyrosine kinase that phosphorylates these tyrosine(s), since receptors lacking these tyrosines are phosphorylated to a significantly reduced extent (full-length) or not at all (truncated receptor) compared with the same sized receptors containing these tyrosines. Presumably, this is the GH-associated JAK2 tyrosine kinase that phosphorylates these tyrosine(s), since the truncated receptor is phosphorylated when GH-GHR<sub>1–454</sub> complexes are precipitated with αGH and incubated with [γ<sup>32</sup>P]ATP, whereas the truncated receptor lacking Tyr<sub>333</sub> and Tyr<sub>338</sub> is not. Although Tyr<sub>333</sub> seems the most likely candidate based upon sequence analysis, additional studies will be required to determine which of the two tyrosines (333 or 338) is phosphorylated. Whether or not these are the only tyrosines in GHR<sub>1–454</sub> that are phosphorylated is not known, since it is possible that phosphorylation of Tyr<sub>333</sub> and/or Tyr<sub>338</sub> is required for the subsequent phosphorylation of Tyr<sub>391</sub> and Tyr<sub>437</sub>. Although it seems unlikely given the conservative nature of the amino acid substitution, our results cannot rule out the alternative possibility that Tyr<sub>333</sub> and Tyr<sub>338</sub> are not themselves phosphorylated but rather, mutating Tyr<sub>333</sub> and Tyr<sub>338</sub> to Phe alters the ability of Tyr<sub>391</sub> and/or Tyr<sub>437</sub> to be phosphorylated.

Tyr<sub>333</sub> and/or Tyr<sub>338</sub> appear not to be the only tyrosines phosphorylated in response to GH, since GH appears to stimulate the tyrosyl phosphorylation of the full-length, Tyr<sub>333</sub> and Tyr<sub>338</sub> mutated receptor. The 6 tyrosines between amino acids 454–638 are the most likely candidates because tyrosines other than 333 and/or 338 present in GHR<sub>1–454</sub> appear not to be phosphorylated to any great extent. Multiple phosphorylated tyrosines in GHR<sub>1–454</sub> appear not to be phosphorylated to any great extent. Multiple phosphorylated tyrosines in GHR would be consistent with multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.

The finding that Tyr<sub>333</sub> and/or Tyr<sub>338</sub> are likely to be phosphorylated in response to GH raises the question of whether either or both serve as binding sites for specific SH2 domains. Neither tyrosine, with its surrounding amino acids, closely resembles a high affinity binding site (as currently defined) of various SH2 domains (reviewed in Ref. 1). Multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.

The finding that Tyr<sub>333</sub> and/or Tyr<sub>338</sub> are likely to be phosphorylated in response to GH raises the question of whether either or both serve as binding sites for specific SH2 domains. Neither tyrosine, with its surrounding amino acids, closely resembles a high affinity binding site (as currently defined) of various SH2 domains (reviewed in Ref. 1). Multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.

The finding that Tyr<sub>333</sub> and/or Tyr<sub>338</sub> are likely to be phosphorylated in response to GH raises the question of whether either or both serve as binding sites for specific SH2 domains. Neither tyrosine, with its surrounding amino acids, closely resembles a high affinity binding site (as currently defined) of various SH2 domains (reviewed in Ref. 1). Multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.

The finding that Tyr<sub>333</sub> and/or Tyr<sub>338</sub> are likely to be phosphorylated in response to GH raises the question of whether either or both serve as binding sites for specific SH2 domains. Neither tyrosine, with its surrounding amino acids, closely resembles a high affinity binding site (as currently defined) of various SH2 domains (reviewed in Ref. 1). Multiple sites of phosphorylation in receptors with intrinsic tyrosine kinase activity (e.g., receptors for insulin, epidermal growth factor, platelet-derived growth factor) (reviewed in Ref. 1). Multiple sites of phosphorylation with differing affinities for various SH2 domains would provide a mechanism by which GH could initiate several signaling pathways simultaneously.
Phosphorylation of Tyr\textsuperscript{333} and/or Tyr\textsuperscript{338} of GHR

31. Billestrup, N., Moldrup, A., Serup, P., Mathews, L. S., Norstedt, G., and Nielsen, J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7210–7214
32. Wang, X., Uhler, M., Billestrup, N., Norstedt, G., Talamantes, F., Nielsen, J. H., and Carter-Su, C. (1992) J. Biol. Chem. 267, 17390–17396
33. Campbell, G. S., Christian, L. J., and Carter-Su, C. (1993) J. Biol. Chem. 268, 7427–7434
34. Foster, C. M., Shafer, J. A., Roza, F. W., Wang, X., Lewis, S. D., Renken, D. A., Natale, J. E., Schwartz, J., and Carter-Su, C. (1988) Biochemistry 27, 326–334
35. Wang, X., Moller, C., Norstedt, G., and Carter-Su, C. (1993) J. Biol. Chem. 268, 3573–3579
36. Carter-Su, C., Stubbart, J. R., Wang, X., Stred, S. E., Argetsinger, L. S., and Shafer, J. A. (1989) J. Biol. Chem. 264, 18654–18661
37. Li, C. H. (1982) Mol. Cell. Biochem. 46, 31–41
38. Campbell, G. S., Pang, L., Miyasaka, T., Saltiel, A. R., and Carter-Su, C. (1992) J. Biol. Chem. 267, 6074–6080
39. Winston, L. A., and Bertics, P. J. (1992) J. Biol. Chem. 267, 4747–4751
40. VanderKuur, J., Allevato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1995) J. Biol. Chem. 270, 7587–7593
41. Wang, Y.-D., Wong, K., and Wood, W. I. (1995) J. Biol. Chem. 270, 7021–7024
42. Lobie, P. E., Allevato, G., Nielsen, J. H., Norstedt, G., and Billestrup, N. (1995) J. Biol. Chem. 270, 21745–21750