Identification of Growth Hormone Receptor (GHR) Tyrosine Residues Required for GHR Phosphorylation and JAK2 and STAT5 Activation

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To determine whether GH receptor (GHR) cytoplasmic tyrosine residue(s) and tyrosine phosphorylation are required for signal transduction, we have substituted the eight porcine (p) GHR cytoplasmic tyrosines with phenylalanine individually or in a stepwise manner from the C terminus. Conversely, the eight tyrosines were individually regenerated in a non-tyrosine-containing pGHR analog. Mutated pGHR cDNAs were transfected into mouse L cells (MLCs) and cell lines were established. Each individual tyrosine-substituted pGHR analogs was able to activate STAT5 (signal transducer and activator of transcription 5; previously termed pp95) at levels comparable to those of wild type pGHR. Analyses of these pGHR analogs revealed that a single tyrosine residue at position 487, 534, 566, or 627 is sufficient for STAT5 phosphorylation. This result suggested that a redundancy in tyrosine residue requirement may be employed in GH-mediated signal transduction. Also, we found that the requirement of tyrosine residues for STAT5 phosphorylation directly correlated with their phosphorylation status. Combining both STAT5 and GHR tyrosine phosphorylation results, we have deduced that Y332, Y487, Y534, Y566, and Y627 are pGHR tyrosine phosphorylation sites. Additionally, Janus kinase 2 was activated by GH in all pGHR tyrosine-substituted analogs, including one containing no intracellular tyrosines, which agrees with a previous report that Janus kinase 2 activation is independent of GHR tyrosine phosphorylation. (Molecular Endocrinology 10: 1249–1260, 1996)

INTRODUCTION

GH induces a variety of metabolic effects after binding to its receptor (GHR). The initial steps in this GH-specific signal transduction pathway include activation of a GHR-associated tyrosine kinase, Janus kinase 2 (JAK2) (1), tyrosine phosphorylation of GHR (2), induction of mitogen-activator protein (MAP) kinases (3–6), and tyrosine phosphorylation of other cytosolic proteins (7–9) including the signal transducers and activators of transcription [STAT 1 (10–12), STAT3 (13), STAT5 (14)], and insulin receptor substrate-1 (IRS-1) (15). Also, activation of protein kinase C (16–19) has been determined to be involved in GH signal transduction pathways.

Although the molecular linkage between the GHR and signal mediators remains unclear, relationships are gradually being established among the elements and motifs on the GHR cytoplasmic domain and the resultant intracellular biological responses. It has been demonstrated that a proline-rich region composed of eight amino acids of the GHR cytoplasmic domain (box 1) is the site where JAK2 associates with the GHR (12, 20–23). Box 1 has been shown to be required for transcription of GH-induced genes, such as the serine protease inhibitor gene (Spi 2.1) and the ovine β-lactoglobulin gene (24). By transfection of a series of GHR truncation analogs into an interleukin-3-dependent promyeloid cell line, FDC-P1, it was shown that these cells not only became GH dependent but also responded to GH via a GHR truncation analog that possessed only box 1 (25). Also, box 1 has been shown to be essential and sufficient for GH-induced activation of JAK2, MAP kinases (22), the JAK2-STAT1-signaling pathway, and proliferation of Ba/F3 cells transfected with GHR (12). However, accumulating data suggest that box 1 alone is not sufficient to transduce all of the GH-specific signals. For example, GH-induced activation of Spi-reporter gene is dependent upon both an intact box 1 as well as an 80- amino acid segment on the C terminus of GHR (22, 23). Also, GH-induced tyrosine phosphorylation of cytoplasmic proteins of molecular masses of ~95/96 kDa is dependent on another region of the GHR cytoplasmic domain, termed box 3 (26). Additionally, it has been shown that a 52-amino acid domain of the GHR cytoplasmic portion is required for GH-induced intracellular free Ca^{2+} oscillations that are independent of box 1 (23). Some

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of these domains may overlap (23, 26, 27). Together, these findings indicate that multiple specific or overlapping domains, which are required for the generation of distinct signals involved in the variety of GH's biological functions, may exist along the cytoplasmic portion of the GHR.

We have previously demonstrated that a complex of cytosolic proteins with a molecular mass of ~95/96 kDa (pp95) can be induced by GH to be tyrosine phosphorylated in mouse L cells (MLCs) that express recombinant GHRs and in mouse 3T3-F442A preadipocytes (9). Using a truncational approach of the GHR, we have determined that a 40-amino acid segment of the GHR is essential for GH-induced tyrosine phosphorylation of pp95. This 40-amino acid segment (box 3) is distinct from the box 1 proline-rich region (26). GH-induced tyrosine phosphorylation of a protein with a molecular mass of 95 kDa was also observed in rat adipocytes (15) and in a mouse promyloid cell line, FDC-P1, transfected with GHR cDNA (12). In addition, tyrosine phosphorylation of proteins with similar molecular masses, *i.e.* 93 kDa (7) and 97 kDa (8), was observed in a human lymphocyte line (IM-9) and in Chinese hamster ovary (CHO) cells transfected with a GHR cDNA, respectively. Despite the differences in molecular mass, it has been shown that all of these proteins are GH-inducible for tyrosine phosphorylation and may play an important role in GH signal transduction. It has been demonstrated that pp95 is not immunologically related with STAT1, -2, -3, and -4 proteins using specific STAT antibodies (28, 29). STAT5 (mammary gland factor) has been identified to be tyrosine phosphorylated after GH treatment and is able to activate gene transcription in CHO cells transfected with a GHR cDNA, as well as *in vivo* (14, 30). We have used STAT5 antibodies to demonstrate that STAT5 is a major component of the GH-induced protein complex pp95/96 (Fig. 1; Ref. 31), which we have described previously (9). Throughout this manuscript we will refer to the pp95/96 complex as STAT5. Regardless of the subtle differences in molecular mass of these proteins, these studies together provide evidence that a class of 90- to 97-kDa proteins play important roles in GH signal transduction.

Although tyrosine phosphorylation of the GHR as well as other cytosolic proteins has been extensively described, the importance and function of individual tyrosine residues in the GHR cytoplasmic domain remain unclear. To gain further insight into the importance of GHR tyrosines in the GH-signaling pathway and establish relationships among GHR tyrosine phosphorylation, STAT5 phosphorylation, and JAK2 activation, we have substituted the eight intracellular pGHR tyrosine residues (Y332, Y337, Y390, Y487, Y534, Y566, Y595, and Y627) with phenylalanine, both individually and in combination. Six of the eight tyrosines are conserved among all reported mammalian GHR sequences (Y332, Y487, Y534, Y566, Y595, and Y627). The mutated pGHR cDNAs were transfected into MLCs, and cell lines were established. Subsequently, the ability of GH to induce tyrosine phosphorylation of the pGHR analogs, STAT5, and JAK2, was examined.

RESULTS

GH-Induced pp95/96 Migrate with Tyrosine-Phosphorylated STAT5 Proteins

Serum-depleted pGHRwt/Neo cells were treated with pGH (500 ng/ml) for 10 min. Proteins in the whole cell lysates (~10 μg of cellular proteins) were separated by 4–12% gradient PAGE, transferred to nitrocellulose membrane, and probed with phosphotyrosine antiserum (Fig. 1, lanes 1 and 2). Elevated levels of pp95/96 tyrosine phosphorylation were observed in GH-treated pGHRwt/Neo cell lysate (Fig. 1, lane 2). One milliliter of cell lysate from GH-treated pGHRwt/Neo cells (containing ~500 μg cellular protein) was subjected to immunoprecipitation (IP) three consecutive times. The original cell lysate (Fig. 1, lane 3) and

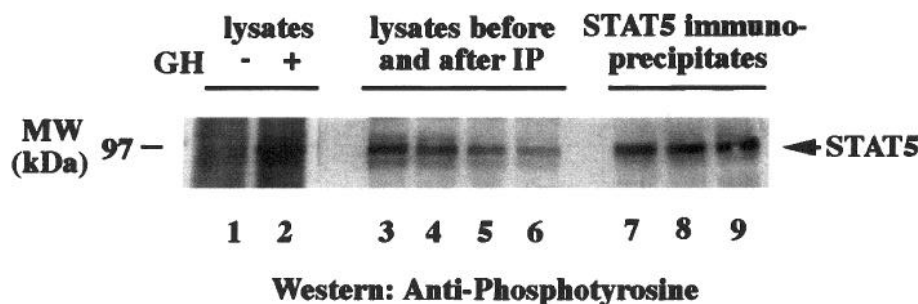

Fig. 1. STAT5 Immunoprecipitated from pGHRwt/Neo Cells Are Tyrosine Phosphorylated and IP Using STAT5 Antiserum Reduces the pp95/96 Signal in GH-Treated pGHRwt/Neo Cell Lysate

Serum-depleted pGHRwt/Neo cells were treated with pGH (500 ng/ml) for 10 min. Proteins in the whole cell lysates were subjected to Western blot analyses using phosphotyrosine antiserum (lanes 1 and 2). IP procedures were performed to the same GH-treated pGHRwt/Neo cell lysate three consecutive times. The original cell lysate (lane 3) and cell lysate after each IP (lanes 4–6) were subjected to Western blot using phosphotyrosine antiserum. Immuno-complexes resulting from each IP were also subjected to Western analyses (lanes 7–9).

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cell lysate after each IP (Fig. 1, lanes 4, 5, and 6) were subjected to Western blot analyses using phosphotyrosine antiserum. The pp95/96 signal after the second round (Fig. 1, lane 5) and the third round (Fig. 1, lane 6) of IP with STAT5 antiserum was decreased compared with that in the original GH-treated pGHRwt/Neo lysate (Fig. 1, lane 3). Also, the immuno-complexes resulting from each IP were subjected to Western analysis using phosphotyrosine antiserum. All three STAT5 immuno-complexes were tyrosine phosphorylated (Fig. 1, lanes 7–9). The pp95/96 band migrates with the same apparent molecular mass as the protein immunoprecipitated with STAT5 antibody. We have recently confirmed that pp95/96 and STAT5 are the same protein (31).

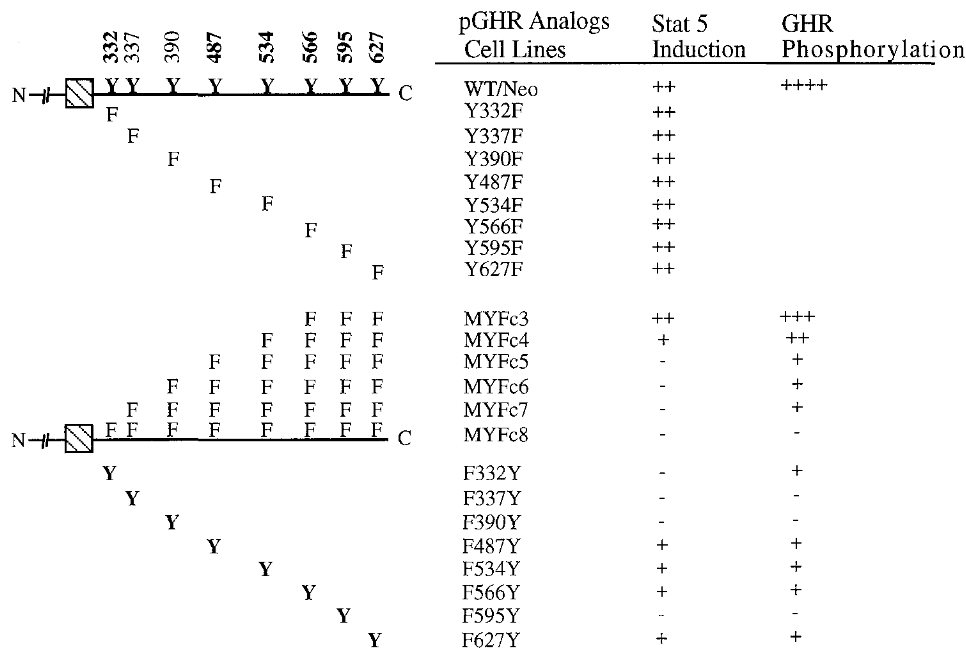
Characterization of Cell Lines Expressing GHR Tyrosine-Substituted Analogs

MLCs, co-transfected with pMet-IG-pGHR, pGHR-YxxxFs, pGHR-MYFcs, or pGHR-FxxxYs and pSV40-Neo (see *Materials and Methods*) were cultured in medium containing the neomycin analog G418, and resistant colonies were pooled for subsequent analyses. MLCs transfected with pSV40-Neo (MLC/Neo) alone served as a negative control and pMet-IG-pGHR (pGHRwt/Neo) served as a positive control. Other cell lines were termed Y332F, Y337F, Y390F, Y487F, Y534F, Y566F, Y595F, Y627F, MYFc3, MYFc4, MYFc5, MYFc6, MYFc7, MYFc8, F337Y, F390Y, F487Y, F534Y, F566Y, F595Y, and F627Y (Fig. 2). Northern blot analyses of total RNA extracted from these cell lines showed that all express the pGHR cDNA or its mutated forms at similar levels (data not shown). In contrast, no signal was observed in MLC/Neo cells.

Receptor-binding assays were performed to evaluate the ability of pGHR or its tyrosine-substituted analogs to bind to pGH. The results are summarized in Fig. 3. pGHRwt/Neo as well as all of the pGHR analog cell lines showed a comparable amount of [^{125}I]pGH specific binding. As was expected, substitution of phenylalanine for tyrosine(s) in the pGHR intracellular domain did not affect binding. No specific binding was detected in MLC/Neo cells. Therefore, all of the cell lines expressed a comparable number of GH receptors except MYFc8, a pGHR analog that contained no tyrosine residues. This cell line had elevated GH-binding capacity compared with pGHRwt/Neo and the other pGHR analog cell lines (Fig. 3).

Y487, Y534, Y566, or Y627 is sufficient for GH-stimulated STAT5 Tyrosine Phosphorylation

To examine the effects of pGHR tyrosine substitutions on their ability to transduce a GH-specific signal, STAT5 tyrosine phosphoylation assays (formerly called pp95 assays) were performed as previously described (9). The results are shown in Fig. 4. The levels of GH-induced STAT5 phosphorylation were similar in



pGHR Analogs Cell Lines	Stat 5 Induction	GHR Phosphorylation
WT/Neo	++	++++
Y332F	++	
Y337F	++	
Y390F	++	
Y487F	++	
Y534F	++	
Y566F	++	
Y595F	++	
Y627F	++	
MYFc3	++	+++
MYFc4	+	++
MYFc5	-	+
MYFc6	-	+
MYFc7	-	+
MYFc8	-	-
F332Y	-	+
F337Y	-	-
F390Y	-	-
F487Y	+	+
F534Y	+	+
F566Y	+	+
F595Y	-	-
F627Y	+	+

Fig. 2. Summary of Tyrosine Substitution pGHR Analogs

The letters, N and C, represent the amino and carboxyl terminus of the pGHR, respectively. The transmembrane domain of GHR is shown as *hatched boxes*. The position of tyrosine (Y) in the pGHR cytoplasmic domain as well as the corresponding phenylalanine (F) substitution is indicated by the *numbers above the schematic* representation of pGHR. The *numbers in bold* represent the position of conserved tyrosines. STAT5 and GHR phosphorylation results were summarized as: ++++, equivalent to wild type pGHR; +++ to +, decreased levels compared with wild type; −, basal levels induction of STAT5, which is equivalent to parental MLC.

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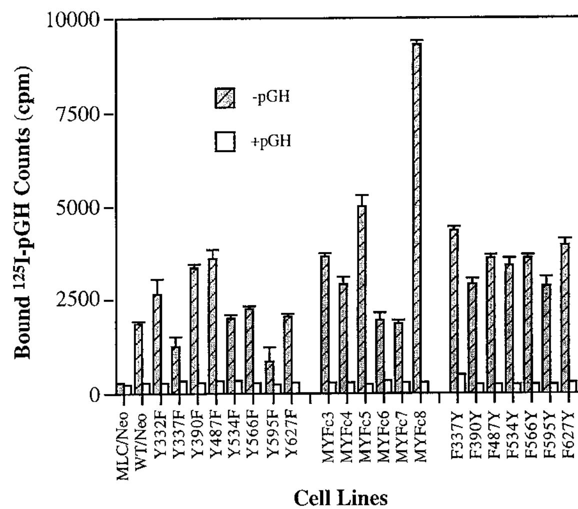

Fig. 3. Maximum Displacement Experiments on Parental MLCs, Stable Cell Lines That Express Wild Type GHR (pGH-Rwt/Neo) or Its Tyrosine Substitution Analogs

Cells were incubated in serum-free medium overnight before the assay was performed. Approximately 60,000 cpm of [^{125}I]pGH were incubated with the cells in the presence or absence of excess unlabeled pGH. The cells were washed and harvested. The radioactivity of each sample was determined. Each value represents the results from three experiments.

pGHRwt/Neo cells (Fig. 4, A-C) and in Y332F, Y337F, Y390F, Y487F, Y534F, Y566F, Y595F, and Y627F cells (Fig. 4A). In contrast, no detectable STAT5 phosphorylation was observed in non-GH-treated cells (Fig. 4A). The basal level of STAT5 phosphorylation in GH-treated MLC/Neo cells is also shown in Fig. 4.

In the stepwise tyrosine-substituted pGHR analogs, GH-induced STAT5 phosphorylation was observed at similar levels in MYFc3 and pGHRwt/Neo cells (Fig. 4B). A decreased level (~one-half) of GH-induced STAT5 phosphorylation was observed in MYFc4 cells relative to pGHRwt/Neo cells (Fig. 4B). Basal levels of GH-induced STAT5 phosphorylation were observed in GH-treated MYFc5, MYFc6, MYFc7, and MYFc8 cells (Fig. 4B). These results indicate that tyrosines 332, 337, 390, and 487 (MYFc4) are sufficient for STAT5 phosphorylation, and removal of Y487 (MYFc5) resulted in the inability of GH to induce STAT5.

Individual tyrosines were replaced in MYFc8 (FxxxY), generating FxxxY analogs containing only one intracellular tyrosine residue. Surprisingly, cells expressing pGHR analogs F487Y, F534Y, F566Y, or F627Y were rescued in their ability to respond to GH by inducing STAT5 phosphorylation. The levels of STAT5 phosphorylation were comparable to those of the pGHR-wt/Neo cells (Fig. 4C). However, replacement of tyrosine at positions 332 (which is equivalent to the pGHR MYFc7 analog), 337, 390, or 595 did not rescue the STAT5 response. GH treatment of these cell lines resulted in basal levels of STAT5 phosphorylation, which is similar to those of the negative control MLC/Neo cells (Fig. 4C).

Together, the results of STAT5 phosphorylation assays in these cell lines indicate that individual tyrosine substitutions in the pGHR cytoplasmic domain do not affect GH-induced STAT5 tyrosine phosphorylation. Substitution of three C-terminal tyrosines with phenylalanines resulted in GHR analogs that retained the ability to stimulate STAT5 at levels comparable to those observed in pGHRwt/Neo cells. Substitution of four C-terminal tyrosines with phenylalanines (MYFc4) resulted in analogs that retained the ability to induce STAT5, but at a decreased level. However, substitution of five or more C-terminal tyrosines with phenylalanines (MYFc5, MYFc6, MYFc7, MYFc8) resulted in cells in which GH-induced STAT5 phosphorylation was not detectable. In addition, the existence of a single tyrosine residue at position 487, 534, 566, or 627 (F487Y, F534Y, F566Y, and F627Y) was sufficient for GH-induced STAT5 tyrosine phosphorylation.

GHR Tyrosine Phosphorylation Is Not Required for JAK2 Activation, and Y332, Y487, Y534, Y566, and Y627 are Phosphorylation Sites

To examine the tyrosine phosphorylation status of these pGHR analogs and to determine the relationship between GHR tyrosine phosphorylation and the ability to induce STAT5 and activate JAK2, GHR analogs and JAK2 proteins were immunoprecipitated and subjected to Western analyses employing a phosphotyrosine antibody. First, we performed these assays on MLC/Neo, pGHRwt/Neo, and MYFc8 cell lines. The native pGHR was intensively tyrosine phosphorylated after GH treatment in pGHRwt/Neo cells (Fig. 5A). In contrast, no signal above the basal level was detected in GH-treated MLC/Neo or MYFc8 cells (Fig. 5A). Interestingly, JAK2 was tyrosine phosphorylated in both pGHRwt/Neo and MYFc8 cells after GH treatment (Fig. 5A). No JAK2 tyrosine phosphorylation over background levels was observed in MLC/Neo cells (Fig. 5A).

To confirm that the pGHR tyrosine phosphorylation signals observed in pGHRwt/Neo were indeed phosphorylated pGHR and not phosphorylated JAK2 or other co-immunoprecipitated proteins, the Western blots were cross-immunoblotted with JAK2 and GHR antiserum. Results are shown in Fig. 5, B and C. JAK2 was detected in anti-JAK2 immunoprecipitates regardless of GH treatment and was not found in immunoprecipitates using GHR antiserum. Thus, the phosphorylation signal observed at approximately 120 kDa is due to the phosphorylation of pGHR or its analogs and is not a result of co-IP of tyrosine-phosphorylated JAK2.

Similar experiments were performed using pGHR-MYFcx cell lines. The results demonstrated that all of the pGHR tyrosine substitution analogs retained the ability to activate JAK2 (Fig. 6). Also, cross-immunoblotting with GHR and JAK2 antiserum confirmed that the tyrosine phosphorylation signals

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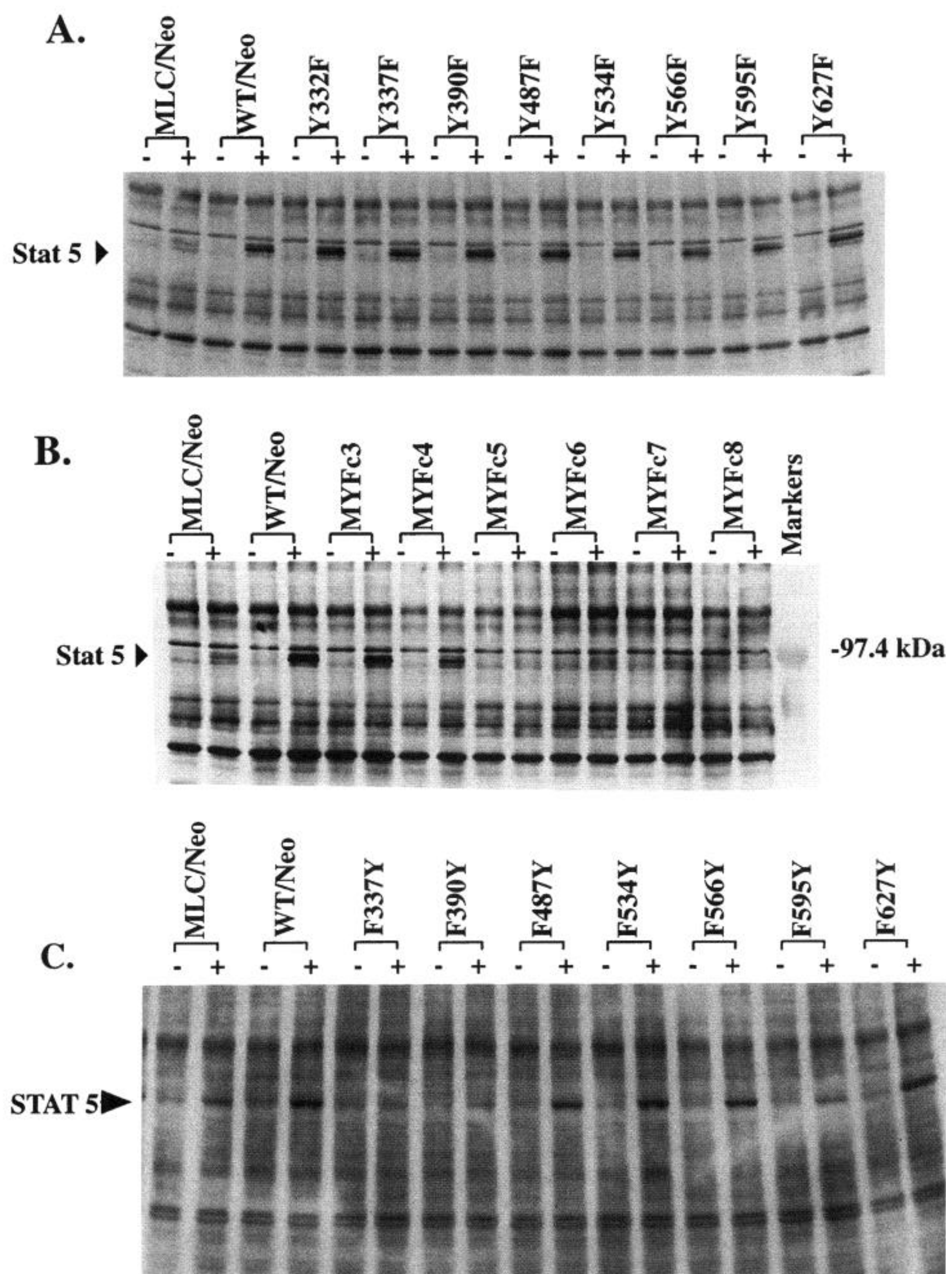

Fig. 4. STAT5 Phosphorylation Assay on Cell Lines That Express Wild Type pGHR or Its Tyrosine Substitution Analogs
The assay was performed as described in *Materials and Methods*. The *arrow* on the *left* indicates the position of STAT5. A, STAT5 phosphorylation in pGHR-YxxxF cells. B, STAT5 phosphorylation in pGHR-MYFcx cells. C, STAT5 phosphorylation in pGHR-FxxxY cells.

were not due to co-IP of either JAK2 or GHR (data not shown).

GHR phosphorylation results using MYFcx cell lines (Fig. 6) revealed that all of the pGHR-MYFcx analogs were phosphorylated after GH treatment, except MYFc8. However, the sequential phosphorylation intensities are as follow: pGHRwt > MYFc3 > MYFc4 > MYFc5 = MYFc6 = MyFc7 (Fig. 6). The decrease in phosphorylation intensity of MYFc3 from wild type pGHR suggests that Y566, Y595, and Y627 are potential phosphorylation sites. In the same scenario, these data suggest that Y534 and Y487 are "potent" phosphorylation sites. Since F332Y is equivalent to MYFc7, it is reasonable to deduce that Y332F is phosphorylated (Fig. 6). Together, these results indicate that tyrosine residues at positions 332, 487, 534, 566, 595, and 627 are phosphorylated. However, considering the STAT5 phosphorylation results (above), we ruled out Y595 as a potential site for phosphorylation (see *Discussion*). The GHR phosphorylation results are summarized in Fig. 2.

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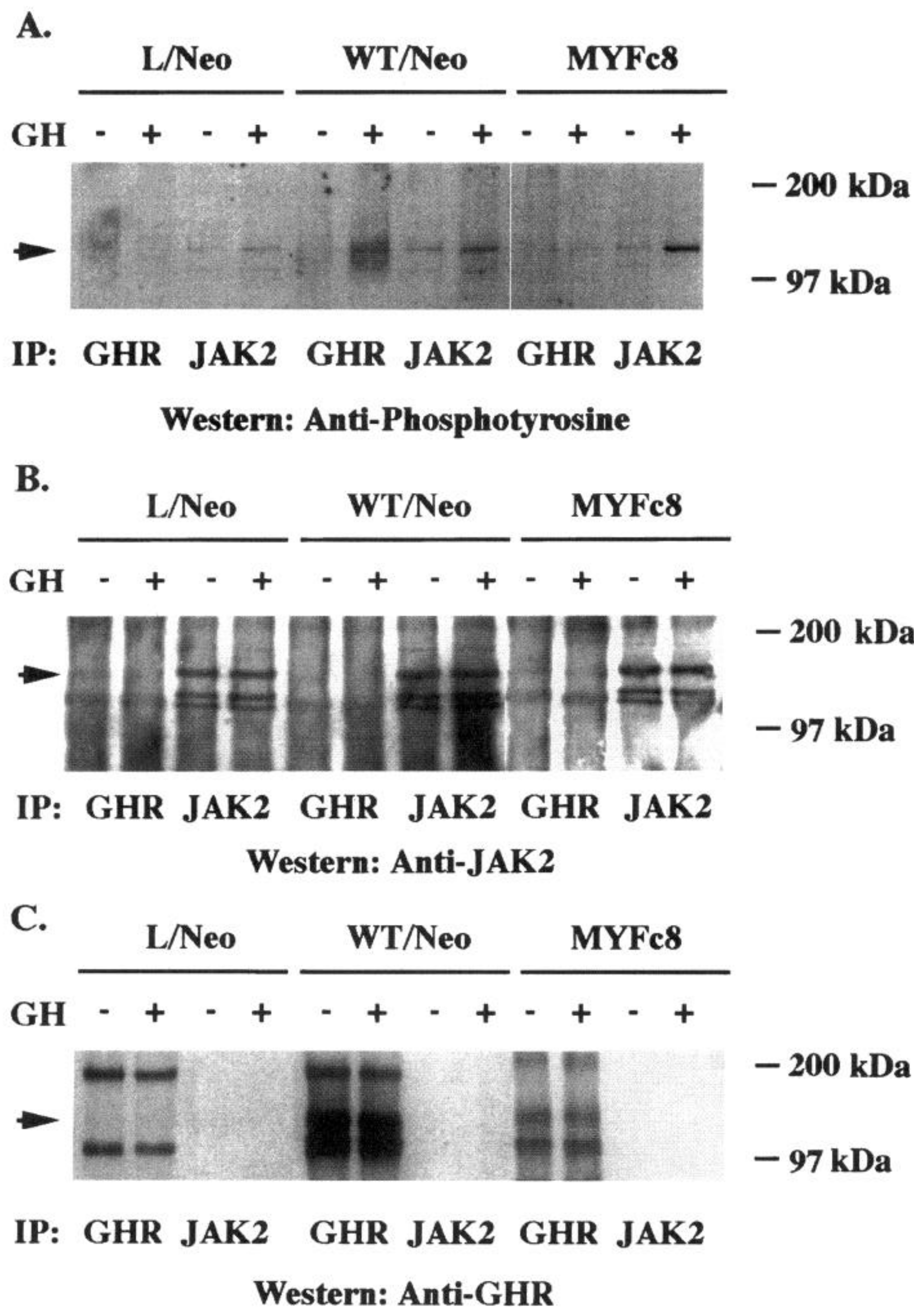

Fig. 5. pGHR Tyrosine Phosphorylation and JAK2 Activation in Cells Expressing Wild Type pGHR or pGHR-MYFc8 Analog

The immunoprecipitates (as described in *Materials and Methods*) were resolved on 7.5% SDS-PAGE, and the blots were probed with: A, horse radish peroxidase-conjugated phosphotyrosine antibody (PY20); B, JAK2 antibody; C, antibody directed against rabbit GHR cytoplasmic domain. The *arrow* on the *left* indicates the position of GHR and JAK2. Panels B and C are cross-immunoblots to demonstrate the phosphorylation signals in panel A are not from cross-contamination of pGHR and JAK2.

DISCUSSION

Expression of pGHR and Tyrosine-Substituted Analogs in MLCs

Using an oligonucleotide-directed mutagenesis approach, the eight pGHR intracellular tyrosine codons were changed to those encoding phenylalanine. These mutated pGHR analogs were expressed in MLCs. Northern analyses and receptor-binding assays showed that pGHR and its analogs were expressed in the transfected MLCs at comparable levels (Fig. 3). Surprisingly, cells that expressed MYFc8 possessed more GH-specific binding sites on the cell surface than the other cell lines, which may be indicative of a defect in receptor internalization. This hypothesis was supported by Northern analyses of RNA isolated from these cell lines. No significant differences were found

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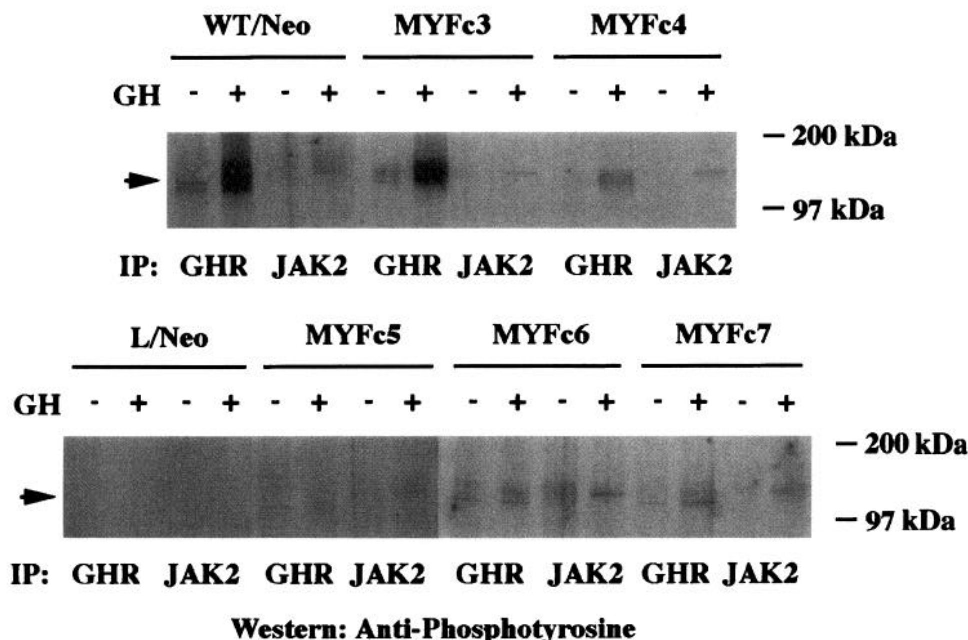

Fig. 6. pGHR Tyrosine Phosphorylation Assays on pGHR-MYFcx Cells
Immunoprecipitates from GHR or JAK2 antiserum were resolved on 7.5% SDS-PAGE gels, and the blots were probed with phosphotyrosine antibody, PY20. The detailed procedure of the pGHR tyrosine phosphorylation assay is described in *Materials and Methods. Arrow on the left* indicates the position of phosphorylated GHR molecules.

in the levels of pGHR RNA. In addition, we have previously demonstrated that a pGHR cytoplasmic domain truncation analog, pGHR-TR1, which contains only three intracellular amino acids, accumulates GH-binding sites on the cell surface (26). These results suggest that tyrosines in the cytoplasmic domain of the GHRs may be important elements in regulating recycling of GHRs in these cells. Additionally, other residues in the intracellular domain of the GHR may be important for internalization. For example, F346 of the rat GHR has been found to be required for internalization (27). Further work is required to define the precise amino acids or motifs necessary for GH/GHR internalization.

Role of pGHR Cytoplasmic Tyrosine Residues in GH-Induced STAT5 Phosphorylation

GH receptors belong to a superfamily of receptors for a variety of hormones, cytokines, and growth factors (32). The family members share many common characteristics, such as ligand-induced receptor dimerization, lack of intrinsic tyrosine kinase activity, conserved extracellular cysteine residues, and other functional motifs (25, 33, 34). The initial cellular response after hormone or growth factor treatment is tyrosine phosphorylation of the receptors themselves and other cytosolic proteins including those of the STAT family and MAP kinases (35). Tyrosine phosphorylation of GHR and other cytosolic proteins after GH treatment has also been described in various systems and have been speculated to play important roles in GH/GHR signaling (1, 6, 8, 9, 29). The most C-terminal tyrosine residue in the rat PRL receptor, a member of the cytokine superfamily of receptors, has been shown to be critical for PRL-induced signal transduction. Replacement of this tyrosine with phenylalanine was found to be detrimental to PRL-activated gene transcription (36). Recently, it has been shown that tyrosines 333 and/or 338 of the rat GHR are phosphorylated and are important for GH-dependent lipogenesis and protein synthesis (37, 38). However, the relationship(s) among most GHR tyrosine residues, their phosphorylation status, and their biological function in GH-GHR signal transduction pathway(s) is not clear.

The role of individual tyrosine residues in epidermal growth factor, platelet-derived growth factor (PDGF), and insulin receptor signal transduction or receptor modulation has been well studied (39, 40). For example, a single tyrosine mutation of the PDGF receptor (Y751 or Y857) can cause dramatic changes in PDGF-mediated signal transduction (41). Primarily, these phosphorylated tyrosines provide docking sites for signal mediators to receive the signal transduced through the cell membrane. A number of protein molecules bearing a *Src* homology (SH2) domain have been shown to interact with tyrosine residues along the receptor cytoplasmic domain (42). Although the GH/PRL receptor family does not behave like growth factor receptors, common events occur after binding to their ligands; *e.g.* activation and subsequent tyrosine phosphorylation of the GHR and other cytosolic proteins. Therefore, it is reasonable to propose that

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the tyrosine residues of GHR cytoplasmic domain may employ a similar mechanism by which the GH signal is transduced.

In this study, we used STAT5 phosphorylation as an assay for screening the biological activity of GHR tyrosine substitutions. Results of the pGHR YxxxF analogs indicated that individual tyrosine substitutions had no effect on GH-dependent STAT5 phosphorylation (Fig. 4A). This result can be explained by at least two hypotheses: 1) The tyrosine residue *per se*, and not its phosphorylation status, is important in GH-mediated signal transduction. 2) More than one tyrosine residue is involved in GHR signal transduction since substitution of any of the tyrosine residues does not completely abolish the GH-induced signal transduction system. The second explanation suggests that several individual tyrosines, or functional redundancy of tyrosine residues, may be important in GH-mediated STAT5 activation.

To test the above hypotheses, we adopted a stepwise mutation strategy to study the roles of "blocks" of tyrosine residues in GH-mediated signal transduction. The tyrosine residues were substituted individually and sequentially from the C terminus of pGHR (*Materials and Methods*). Substitution of the three C-terminal tyrosines (MYFc3) had no effect on STAT5 phosphorylation (Fig. 4B). However, MYFc4 showed decreased levels (~one-half) of STAT5 phosphorylation. Additionally, MYFc5, MYFc6, MYFc7, and MYFc8 lost their ability to induce STAT5 tyrosine phosphorylation (Fig. 4B).

These findings fit well with previous results from our laboratory concerning pGHR truncation studies (26). In these studies we determined that pGHR-TR6, an analog that contains a truncation of amino acids 559–638, had no effect on GH-induced STAT5 tyrosine phosphorylation. This pGHR truncation is equivalent to MYFc3 in terms of tyrosine residues that are absent. Additionally, another pGHR truncation analog, pGHR-TR4, which contained a deletion of amino acids 476–638, was unable to induce STAT5 phosphorylation. pGHR-TR4 results in a deletion of Y487 and Y534, which is equivalent to MYFc5 in terms of tyrosine residues. Thus, STAT5 phosphorylation via MYFcs agrees with the results of pGHR truncation analogs studies. These results have demonstrated that Y487 is essential and, together with Y534, can yield full activity in terms of STAT5 phosphorylation.

Y487 and Y534 are conserved amino acid residues among all GHRs reported, which suggests the importance of these tyrosine residues in GHR function. Therefore, we generated a double pGHR analog, Y487,534F, to examine the importance of these tyrosines in signaling. Surprisingly, pGHR Y487,534F had equivalent activity as the wild type pGHR in the STAT5 phosphorylation assay (unpublished data). This suggested that tyrosine residues at other locations may compensate in the absence of Y487 and Y534. Since this finding supported our tyrosine redundancy hypothesis, we were encouraged to perform more detailed substitution analyses on other pGHR tyrosine residues.

Individual tyrosine residues were individually replaced into the pGHR-MYFc8 analog. STAT5 tyrosine phosphorylation assays revealed that a single tyrosine at position 487, 534, 566, or 627 fully rescued pGHR-MYFc8 STAT5 activity. These results provided additional support for the tyrosine redundancy hypothesis. We have found that not only the presence of a tyrosine residue in the GHR cytoplasmic domain is essential for GH-dependent STAT5 phosphorylation, but also the position of the tyrosine is crucial for connecting the correct signaling network. A similar redundancy has been documented in other cytokine receptors studies, such as gp130 and leukemia inhibitory factor receptor (35). It was suggested that multiple tyrosine residues of the cytokine receptors can provide docking sites for activation of STAT proteins. However, none of the four important pGHR tyrosines share similarity with STAT 3 tyrosine-specific motif(s) (35).

As we observed previously in pGHR truncation studies (26), a dominant negative effect was seen in MYFc5 and MYFc8 cell lines. The basal levels of STAT5 phosphorylation in these lines were suppressed to nondetectable levels (Fig. 4B). In fact, the MYFc8 and MYFc5 cell lines expressed the highest levels of pGHR analogs. These data are consistent with STAT5 phosphorylation requiring two full-length pGHRs in the dimer complex.

JAK2 Activation Is Independent of GHR Tyrosine Phosphorylation

JAK2 activation has been shown to be an early event in GH signal transduction (1). GH-stimulated tyrosine phosphorylation of JAK2 was found in all cells expressing pGHR tyrosine substitution analogs, including pGHR-MYFc8; *i.e.* the analog without intracellular tyrosine residues (Fig. 5A and Fig. 6). This indicates that JAK2 activation is independent of GHR tyrosine phosphorylation. It has previously been demonstrated that a truncated human GHR with its single intracellular tyrosine residue mutated to phenylalanine is able to activate JAK2 (43). Recently, we have shown that STAT5 associates with GHR in a GH-dependent manner (31). Therefore, the absence of phosphotyrosine or STAT5 docking sites in the GHR (pGHR-MYFc8) would abolish GH-induced STAT5 phosphorylation (Fig. 7).

A human GHR analog containing 54 intracellular amino acids was shown to be sufficient for GH-induced JAK2 phosphorylation, STAT1 (p91) activation, and cellular proliferation in Ba/F3 cells (12). Combining those results with the results reported here suggests that phosphorylated JAK2 can trigger two independent STAT-related signaling pathways, *i.e.* one initiated by STAT 1 and the other by STAT5 (Fig. 7). Expansion of this notion leads to the proposal that STAT5 phosphorylation is not required for cellular proliferation, and STAT1 activation does not require the

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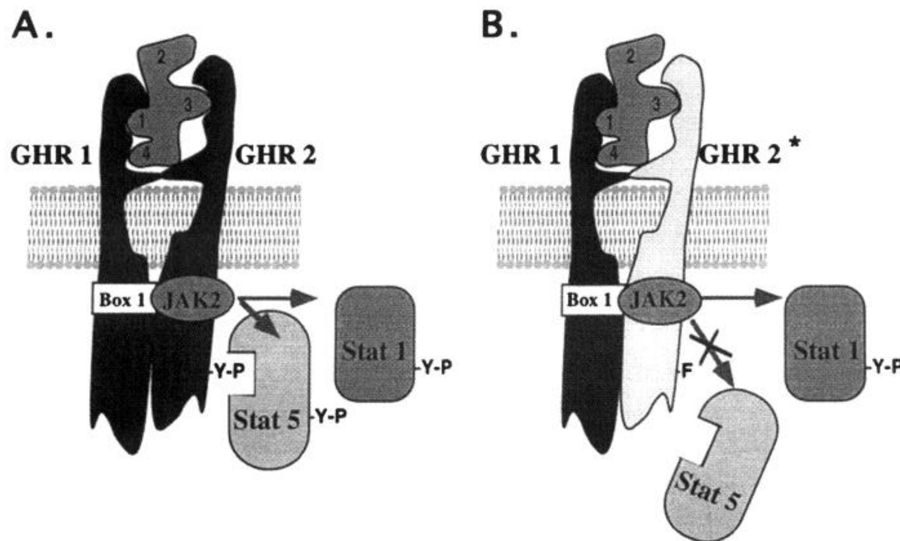

Fig. 7. Hypothetical Model of STAT5 Phosphorylation Pathway

A, Binding of GH to its receptors induces GHR dimerization (GHR1 and GHR2). The *numbers* on the GH molecule indicate the four helices involved in binding. JAK2 associates with the GHRs through the box 1 sequence in the GHR cytoplasmic domain. Activated JAK2 phosphorylates itself as well as the GHR intracellular tyrosines, which provide docking sites for STAT5 association (probably through its SH2 domain). In turn, STAT5 is phosphorylated by JAK2 or other kinases. After being phosphorylated, STAT5 leaves the GHR and continues to transduce signals in the cytoplasm or nucleus. STAT 1 is phosphorylated by JAK2. B, GHR tyrosine substitution eliminates docking sites for STAT5 and prevents STAT5 from being activated by phosphorylation. STAT 1 is phosphorylated by JAK2.

GHR box 3 region. A model that depicts these independent STAT1 and STAT5 tyrosine phosphorylation events is shown in Fig. 7.

Y332, Y487, Y534, Y566, and Y627 are pGHR Phosphorylation Sites

GHR tyrosine phosphorylation was detected in all of the MYFcx mutants except MYFc8. However, the intensity of the phosphorylation signal of wild type pGHR and MYFc3 is much stronger than MYFc4, with a lower level of activity assigned to MYFc5, 6, and 7 (Fig. 6). The dramatic decreased signal intensity of MYFc4 (contains Y627, Y595, Y566, and Y534) relative to MYFc3 (contains Y627, Y595, and Y566) suggested that Y534 is the most abundantly phosphorylated tyrosine residue. The GHR phosphorylation assay also revealed that pGHR-MYFc7 (which contains only Y332) is phosphorylated upon GH treatment. Combining the pGHR-STAT5 phosphotyrosine docking hypothesis and the STAT5 phosphorylation results in pGHR-FxxxY cells has led us to predict that Y627, Y566, and Y487 are also potential phosphorylation sites. Together our results suggest that tyrosine residues 332, 487, 534, 566, and 627 are phosphorylation sites. Interestingly, these five tyrosine residues are conserved in all the GHRs reported to date.

In conclusion, this study indicates that individual tyrosine substitutions had no effect on STAT5 phosphorylation. GH-dependent STAT5 phosphorylation relies on the existence of one tyrosine residue at position 487, 534, 566, or 627. Therefore, GH-dependent STAT5 phosphorylation may utilize redundant tyrosines. We have also demonstrated that JAK2 activation is independent of GHR tyrosine phosphorylation in a full-length pGHR analog lacking intracellular tyrosines. Our results suggest that Y332, Y487, Y534, Y566, and Y627 of pGHR or the corresponding tyrosines of GHRs from other species are phosphorylation sites.

MATERIALS AND METHODS

STAT5 IP

IPs using STAT5 antibody were performed according to a modified procedure suggested by the vendor (Transduction Laboratories, Lexington, KY). GH-treated and control pGH-Rwt/Neo cells were washed with PBS and lysed by adding 1 ml of boiling IP lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4) to each T-75 flask. The cell lysates were transferred to 1.5-ml Eppendorf tubes and boiled for 5 min. Genomic DNA was sheared by forcing cell lysates through 26-gauge needles several times. The lysates were then centrifuged for 5 min, and the supernatants were retained as total cell lysates for IP. Five micrograms of STAT5 monoclonal antibody were mixed with 100 ml of each cell lysate, 400 ml H_2O, and 500 ml of 2× IP buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium ortho-vanadate, 0.4 mM phenylmethylsulfonylfluoride, 1% NP-40) and incubated at 4 C overnight with constant mixing. Five micrograms of polyclonal rabbit anti-mouse IgG antibody were added to each IP reaction and incubated for an additional 1 h. Fifty milliliters of protein A-agarose prewashed in 1× IP buffer were added to each IP reaction. After incubation at 4 C for 2 h, the protein A-agarose beads were washed three times

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with 1× IP buffer. The pellets were resuspended in 50 ml of 1× SDS-PAGE loading buffer, boiled for 5 min, and centrifuged for 5 min. The resulting supernatants were subjected to Western blot analyses using phosphotyrosine antiserum.

Oligonucleotide-Directed Mutagenesis of the pGHR cDNA

The protein-coding region of the pGHR cDNA (EMBL X54429) was inserted into a mammalian expression vector, pMet-IG7, at *Xba*I and *Eco*R I sites. Transcription of pGHR cDNA was directed by the mouse metallothionein I (mMet-I) transcriptional regulatory sequence and used the bovine GH (bGH) gene poly(A) addition signal. The resulting plasmid, which also possessed the bacteriophage f1 intergenic region for production of single-stranded DNA (ssDNA), was termed pMet-IG-pGHR (26). pMet-IG-pGHR ssDNA was prepared as described previously (44).

Eight oligonucleotides (21 mers) were designed at specific positions that convert eight tyrosine codons, TAT or TAC, to phenylalanine, TTT or TTC. For individual tyrosine substitution, one specific oligonucleotide encoding the mutation was used in the reaction as described previously (26, 44). For the stepwise tyrosine substitutions, multiple oligonucleotides corresponding to the desired mutations were used in the reaction. For example, the oligonucleotides encoding the Y627, Y595, and Y566 mutations were used for generating the pGHR analog-Y627,595,566F (MYFc3). The GHR analog terminology refers to the simultaneous mutation (M) of three tyrosines (Y) to phenylalanines (F) from the C terminus (c) of pGHR. Plasmids containing the specific mutations were screened by DNA sequencing (45). Plasmids that contained a single tyrosine-substituted pGHR cDNA were termed pGHR-YxxxF. The xxx indicates the pGHR target tyrosine residue. The plasmids containing multiple tyrosine-substituted pGHR cDNA were termed MYFcx, in which the x represents the number of tyrosines substituted from the C terminus of pGHR. Similarly, seven oligonucleotides (21 mers) were designed at the desired positions to revert the eight phenylalanine codons (TTT or TTC) in MYFc8 to tyrosine codons (TAT or TAC). These mutated pGHR analogs were named pGHR-FxxxY. The tyrosine-substituted pGHR analogs used in this study are summarized in Fig. 2.

Generation of Cell Lines That Express pGHR or pGHR Tyrosine Substitution Analogs

pMet-IG-pGHR, pGHR-YxxxFs, pGHR-MYFcs, and pGHR-FxxxYs cDNAs were generated as described above and were used to establish MLC lines that permanently express pGHR or its analog. MLCs were cotransfected with pGHR or the mutated plasmid DNA and pRSV-Neo DNA as described previously (26). The G418-resistant cells were identified and propagated in tissue culture flasks. These G418-resistant cell pools were maintained in DMEM containing 10% Nu-serum I culture supplement (Becton Dickinson, San Jose, CA), 10 μg/ml gentamicin (Life Technologies, Inc., Gaithersburg, MD) and 200 μg/ml of G418 (Sigma Chemical Co., St. Louis, MO). Total cellular RNA was extracted from each cell line using RNAzol (CINNA/BIOTECX, Houston, TX) (46). Expression of pGHR-YxxxFs, pGHR-MYFcxs, and pGHR-FxxxYs was determined either by RNA slot blotting or Northern analyses using ^{32}P-labeled full-length pGHR cDNA as a hybridization probe (data not shown).

GH Receptor-Binding Assays of the Stable Cell Lines

The GHR analog-expressing cells were plated in six-well tissue culture plates and propagated to 100% confluence (1×10^6 cells per well). Binding assays were performed as previously described (46). Briefly, monolayers were depleted of GH by incubation in serum-free medium (DMEM) for 30 min at 37 C and washed three times in PBS/1% BSA solution for 30 min at room temperature. Approximately 60,000 cpm of [^{125}I]pGH (100 μCi/μg) (a gift from American Cyanamid Company, Princeton, NJ) in PBS/1% BSA were added to each well with or without an excessive amount (2 μg/ml) of nonradioactive pGH (kindly provided by Dr. Shunzhang Qi, Beijing Agricultural University, Beijing, Peoples Republic of China) in a total volume of 1 ml. After incubation for 2 h with gentle agitation at room temperature, the reaction was terminated by rinsing the cells three times with PBS/1% BSA. The cells were then solubilized in 1 ml of 0.1 N NaOH/1% SDS solution and transferred to tubes for γ-count quantification (Beckman Gammma 5500, Beckman Instruments, Fullerton, CA). Specific binding was calculated as the difference between total binding and nonspecific binding [(^{125}I]pGH bound to the cells in the presence of 2 μg/ml of pGH). All determinations were based upon the mean value of three experiments. Each experiment was carried out in triplicate.

STAT5 Phosphorylation Assays in Cells That Express pGHR or Tyrosine-Substituted pGHR Analogs

Cell lines were plated in six-well tissue culture plates and propagated to 95% confluence. GH in the medium was depleted by overnight incubation of cells in serum-free medium (DMEM). Subsequently, a STAT5 phosphorylation assay was performed as described previously (9). Briefly, cells were treated for 10 min at 37 C with or without pGH (500 ng/ml). The cells were lysed in SDS-PAGE lysis buffer, and the cell lysates were subjected to 4%–12% gradient SDS-PAGE. Proteins were transferred to Hybond-ECL membrane (Amersham, Arlington Heights, IL). The membrane was blocked with PBS/4% BSA solution and probed with a phosphotyrosine monoclonal antibody (PY20). The membrane was developed by the conventional ECL procedure (Amersham). The signal was visualized by exposure to x-ray film.

IP of pGHR and JAK2 and Examination of the Tyrosine Phosphorylation status of the pGHR Analogs

The MLC lines expressing pGHR or its tyrosine substitution analogs were seeded into two T-75 tissue culture flasks and grown until confluent ($\sim 1 \times 10^7$ cells per flask). GH in the medium was depleted by incubating the cells for 4 h at 37 C in serum-free medium (DMEM). The cells were then treated for 10 min at 37 C with or without pGH (500 ng/ml). After GH treatment, the cells were washed three times with ice-cold PBS. The cells were then lysed in 3 ml RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), scraped from the plates, and homogenized with a Tenbroeck tissue grinder (10 strokes). The cell lysates were centrifuged at 230,000 × *g* for 1 h at 4 C. The supernatant was incubated at 4 C for at least 2 h with antibody directed against either rat GHR intracellular domain (1:2000, kindly provided by Drs. G. Peter Frick and H. Maurice Goodman, University of Massachusetts Medical School, Worcester, MA) or JAK2 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). Protein A agarose beads (BRL Life Technology Inc., Rockville, MD) were added and the incubation was continued for an additional hour at 4 C. Precipitated complexes were collected by centrifugation and washed three times with RIPA buffer. The pGHR or JAK2 immunoprecipitates were eluted by resuspending the immune complexes in SDS-PAGE loading buffer and resolved by a 7.5% SDS-PAGE. Proteins were transferred to Hybond-ECL membrane. The membrane was blocked with PBS/4% BSA solution and probed with antibody directed against either phosphoty-

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rosine (PY20), rat GHR, or JAK2. The membrane was developed by the conventional ECL procedure (Amersham) and signal visualized by exposure to x-ray film.

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