The role of GH receptor tyrosine phosphorylation in Stat5 activation

J Hansen, L Hansen, X. Wang, J Kopchick, F. Gouilleux, B Groner, J Nielsen,
A Møldrup, E Galsgaard, N Billestrup, et al.

The role of GH receptor tyrosine phosphorylation in Stat5 activation

J A Hansen, L H Hansen, X Wang[1], J J Kopchick[1], F Gouilleux[2], B Groner[2], J H Nielsen, A Møldrup, E D Galsgaard and N Billestrup

Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark

[1]Edison Biotechnology Institute and Department of Clinical Medicine, College of Osteopathic Medicine, Ohio University, Athens, Ohio 45701, USA

[2]Institute for Experimental Cancer Research, Tumor Biology Center, D-79106 Freiburg im Breisgau, Germany

(Requests for offprints should be addressed to N Billestrup)

ABSTRACT

Stimulation of GH receptors leads to rapid activation of Jak2 kinase and subsequent tyrosine phosphorylation of the GH receptor. Three specific tyrosines located in the C-terminal domain of the GH receptor have been identified as being involved in GH-stimulated transcription of the Spi 2·1 promoter. Mutated GH receptors lacking all but one of these three tyrosines are able to mediate a transcriptional response when transiently transfected into CHO cells together with a Spi 2·1 promoter/luciferase construct. Similarly, these GH receptors were found to be able to mediate activation of Stat5 DNA-binding activity, whereas the GH receptor mutant lacking all intracellular tyrosines was not. Synthetic tyrosine phosphorylated peptides corresponding to the GH receptor sequence around the three tyrosines inhibited Stat5 DNA-binding activity while their non-phosphorylated counterparts were ineffective. Tyrosine phosphorylated GST-GH receptor fusion proteins specifically bound to Stat5 in extracts from COS 7 cells transfected with Stat5 cDNA. This binding could be inhibited by tyrosine phosphorylated peptides derived from the GH receptor. This study thus demonstrated that specific GH receptor tyrosine residues, in their phosphorylated state, are involved in transcriptional signaling by directly interacting with Stat5.

Journal of Molecular Endocrinology (1997) **18**, 213–221

INTRODUCTION

The growth hormone (GH) receptor belongs to the cytokine receptor superfamily which is characterized by conserved amino acid sequences in both their extracellular and intracellular domains (Kelly *et al.* 1991). Binding of GH to its receptor results in the association and subsequent activation of the cytoplasmic tyrosine kinase Jak2 (Argetsinger *et al.* 1993). This activation of Jak2 is considered to be required for most signaling pathways mediated by the GH receptor since mutation of the Jak2 binding box-1 region in the membrane proximal domain of the GH receptor interferes with most signaling pathways (Goujon *et al.* 1994, VanderKuur *et al.* 1994, 1995*a*, Allevato *et al.* 1995, Billestrup *et al.* 1995). Upon activation of Jak2 kinase, several proteins become tyrosine phosphorylated, including

the GH receptor itself (Carter-Su *et al.* 1989), Jak2 (Argetsinger *et al.* 1993), mitogen-activated protein (MAP) kinase (Campbell *et al.* 1992, Moller *et al.* 1992), Stat1, 3 and 5 (Campbell *et al.* 1995, Gouilleux *et al.* 1995, Wang *et al.* 1995*b*, Ram *et al.* 1996) as well as an unidentified pp95 protein (Wang *et al.* 1995*a*).

The functional importance of GH receptor tyrosine phosphorylation is beginning to be resolved. We have recently identified tyrosine(s) 333 and/or 338 in the membrane proximal region as being phosphorylated in response to GH (VanderKuur *et al.* 1995*b*). Mutation of these two tyrosines to phenylalanines resulted in a receptor which was defective in mediating GH-stimulated lipogenesis and protein synthesis (Lobie *et al.* 1995). However, these tyrosines were not required for GH receptor internalization or GH-mediated MAP

kinase activation and transcriptional signaling (Lobie *et al.* 1995). Furthermore, it has been shown that a truncated GH receptor lacking all intracellular tyrosines was capable of mediating a mitogenic response when expressed in the promyeloid cell line FDC-PI (Wang *et al.* 1995*b*). This mutated GH receptor also retained its ability to stimulate Jak2 as well as to activate the DNA-binding activity of Stat1, indicating that GH receptor tyrosine phosphorylation is not required for these activities. We have previously identified two domains in the GH receptor required for stimulation of transcriptional signaling (Møldrup *et al.* 1991, Billestrup *et al.* 1995). One domain is the proline-rich box-1 region which is required because of its role in Jak2 binding and the other domain is located within the C-terminal 184 amino acids. In this C-terminal domain we have identified three tyrosine residues at positions 534, 566 and 627 which were found to be involved in transcriptional signaling (Hansen *et al.* 1996). When all three tyrosines were mutated to phenylalanines the receptor could no longer mediate GH activation of the Spi 2·1 promoter. However, any one of these three tyrosines when present as the only intracellular tyrosine was able to mediate GH-stimulated transcription. We found that Stat5 was also not activated by a GH receptor lacking all intracellular tyrosines, which is in contrast to that which has been observed with Stat1 (Wang *et al.* 1995*b*).

In this study we have characterized the specific tyrosine residues in the intracellular domain of the GH receptor which are involved in Stat5 activation and we have examined the ability of GH receptor-derived peptides to interfere with Stat5 binding. In addition, we have tested the ability of Stat5 to directly bind tyrosine phosphorylated GST-GH receptor fusion proteins.

MATERIALS AND METHODS

Plasmids

The plasmids containing the porcine GH receptor cDNA and the RSV-β-galactosidase gene have been described previously (Hansen *et al.* 1996). The Spi 2·1 promoter/luciferase (Spi 2·1-luc) plasmid was constructed by ligating two copies of the XhoI site of the Spi 2·1-GLE I GH-responsive element 5′-tcgaACGCTTCTACTAATCCATGTTCTGA GAAATCATCCAGTCTGCCCA-3′ into the XhoI site of the pGL2-promoter vector (Promega). The E-tag Stat5 expression plasmid (pXM-E-MGF) contains cDNA sequence upstream of the multiple cloning site, this encodes an antigenic determinant derived from the human influenza hemaglutinin

(HA) protein. The expressed HA-Stat5 protein is specifically recognized by the monoclonal anti-HA antibody.

Cell culture and transfection

Chinese hamster ovary (CHO) Kl cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Stably transfected CHO cell lines expressing the various mutated porcine GH receptors were generated as previously described (Hansen *et al.* 1996). Binding experiments were carried out using ^{125}I-labeled human GH (hGH; 20 000 c.p.m.) in 25 mM HEPES (pH 7·4), 124 mM NaCl, 4 mM KCl, 2 mM KH_2PO_4, 1·5 mM $MgCl_2$ and 1 mM $CaCl_2$ as described previously (Hansen *et al.* 1996). Individual clones expressing between 10 000 and 15 000 GH receptors per cell were analyzed. For the luciferase assay, CHO cells were transfected with 1·5 μg Spi 2·1-luc plasmid, 3 μg GH receptor plasmid and 3 μg RSV-β-galactosidase-encoding plasmid using the calcium phosphate precipitation method. After 24 h of culture the cells were stimulated with 20 nM hGH for 6 h. The cells were harvested in cell culture lysis reagent (Promega) and the luciferase activity was measured using the luciferase assay substrate according to the manufacturer's instructions (Promega).

The monkey kidney cell line, COS 7, was cultured in Dulbecco's Modified Eagle's Medium (DMEM; 4500 mg/ml D-glucose; Gibco) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Approximately $2·2 × 10^6$ cells per 150 mm cell culture dish were seeded out and the cells were transiently transfected by a DEAE-dextran/chloroquine method the following day. A DNA precipitate was made by mixing 880 μl 0·1 mM chloroquine, 110 μl 10 mg/ml DEAE-dextran and 110 μl pXM eukaryotic expression vector (11 μg). The medium was changed to DMEM containing 0·1 mM chloroquine. The DNA precipitate was added dropwise and the cells were incubated for 4 h. The medium was removed and the cells were subjected to 10% DMSO (in culture medium) for 1 min. Forty-eight hours following transfection, cell lysates were prepared. Cells ($8–16 × 10^6$) were washed once with ice-cold PBS and lysed by the addition of 2 ml lysis buffer (50 mM HEPES, pH 7·2, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 0·1% NP-40, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride

(AEBSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml herbimycin and 1 mM sodium orthovanadate) followed by 20-min incubation on ice. The cell lysates were centrifuged at 10 000 g for 10 min at 4 °C and supernatants were used for GST binding assays.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were stimulated with 20 nM of hGH for 5 min and washed twice with ice-cold PBS and lysed in a hypotonic buffer (20 mM HEPES, 1 mM EDTA, 1 mM MgCl$_2$, 10 mM KCl and 20% glycerol, 1 mM dithiothreitol, 0·5 mM AEBSF, 1 mM Na$_3$VO$_4$, 1 µg/ml leupeptin and 1 µg/ml aprotinin) containing 0·5% triton X-100. After a 5-min incubation on ice the cells were centrifuged at 2500 g for 7 min at 4 °C. The pellet was resuspended in a hypertonic buffer (hypotonic buffer containing 400 mM NaCl) and incubated on a rocking platform for 30 min on ice. The supernatant was collected after centrifugation at 20 000 g for 30 min at 4 °C. The EMSAs were carried out as previously described (Hansen *et al.* 1996) or pre-incubated with peptides for 60 min at 40 °C, and assayed on 5% native polyacrylamide gel which were exposed to X-ray film. For supershift experiments, the nuclear extract was preincubated with either preimmune serum or Stat5 antiserum for 30 min prior to the addition of probe. The Spi-GLE1 probe 5'-agctATGTTCTGAGAA ATC-3' was labeled and purified as previously described (Hansen *et al.* 1996).

Generation of GST and GST-GH receptor fusion proteins

The cDNA encoding the C-terminal region of the rat GH receptor was amplified by PCR from the plasmid pLM108 which contains the cDNA encoding the full-length GH receptor (Mathews *et al.* 1989). The following primers were used: 5'-oligonucleotide sense primer with a BamHI restriction enzyme site at its 5'-end, 5'-atagggatccCCAAACCACAGCCACTT-3', and an antisense 3'-oligonucleotide primer with an XhoI restriction enzyme site at its 5'-end, 5'-ccgctcgagCTGCATGATTTTGTT-3'. The PCR product was digested with BamHI and XhoI and the BamHI/XhoI fragment encoding the GH receptor amino acid residues 455–638 was ligated into the GST fusion vector pGEX-5X-3 (Pharmacia). The resulting plasmid was sequenced to verify the fidelity of the PCR and to confirm proper, in frame, cloning. Induction and affinity purification of the GST protein and GST-GH receptor (455–638)

fusion proteins were performed as recommended by the manufacturer (Pharmacia). In addition, GST protein and tyrosine phosphorylated GST-GH receptor (455–638) fusion proteins (denoted GST/TKX1 and GST-GH receptor/TKX1) were induced and purified from the *Eschericia coli* TKXL strain that harbors a plasmid encoding an inducible tyrosine kinase gene as recommended by the manufacturer (Stratagene).

GST-GH receptor fusion protein binding assays

Preclearing of cell lysates was performed in microcentrifuge tubes first by binding 300 µg GST/TKX1 protein to 600 µl (50%) glutathione Sepharose 4B (Pharmacia) for 30 min at room temperature followed by four washes with PBS. Cell lysate (1 ml; 4–8 × 10^6 cells) was then added to the tubes which were incubated for 4 h at 4 °C. After centrifugation for 5 min, 250 µl of the supernatants were added to microcentrifuge tubes containing 50 µl (50%) glutathione Sepharose 4B prebound to either 25 µg GST protein or GST-GH receptor (455–638) fusion protein. The tubes were rotated and incubated for 12–14 h at 4 °C. In experiments in which GH receptor peptides were used for competition, the indicated amount of peptide was added to the GST glutathione Sepharose prior to the addition of cell lysate. The resulting Sepharose pellets were washed five times with ice-cold lysis buffer, resuspended in 50 µl SDS-PAGE sample buffer, boiled for 5 min and centrifuged for 5 min. Finally, 40 µl of the supernatants were analyzed by SDS-PAGE followed by Western blotting.

Peptides

Synthetic peptides were purchased from Affinity Research Products Limited either non-phosphorylated or tyrosine phosphorylated. Four, 13 amino acid long peptides derive from the porcine GH receptor were synthesized: FIMDNAYFC EADA (peptide Y534), FNQEDIYITTESL (peptide Y566), EMPVDPYTSIHIV (peptide Y595) and FLSSCGYVSTDQL (peptide Y627). The peptides were purified by HPLC and verified by mass spectroscopy.

Western blot analysis

Proteins were separated by SDS-PAGE (4% stacking gel, 7·5% separating gel) and transferred by electroblotting to ECL nitrocellulose membranes (Amersham International plc, Amersham, Bucks,

UK). The membranes were blocked for 1 h in TBST buffer (50 mM Tris–HCI, pH 7·4, 15 mM NaCl and 0·1% Tween 20) containing 5% non-fat dry milk (1% BSA when membranes were used for anti-phosphotyrosine Western blot). The membranes were washed four times with TBST before the primary antibody diluted in TBST (with 0·2% gelatine) was added (monoclonal anti-phosphotyrosine antibody, 4G10; Upstate Biotechnology; 1:1500 dilution and monoclonal anti-HA, 12CA5; Boehringer Mannheim, Germany; 1:250 dilution) and incubated for 1 h at room temperature. After three successive 20-min washes with TBST the secondary antibody (sheep anti-mouse Ig, horseradish peroxidase-linked whole antibody, NXA 931; Amersham International plc; 1:5000 dilution) was added and the membranes were incubated for an additional 1 h. Following three further 20-min washes with TBST, the proteins were visualized by the ECL detection system according to the manufacturer's instructions (Amersham International plc). Rainbow colored protein molecular weight markers (Amersham International plc) were used to determine molecular weights.

RESULTS

The relative importance of GH receptor tyrosines in transcriptional signaling was determined from transient transfection assays using either an Spi 2·1 promoter/CAT construct (Hansen *et al.* 1996) or an Spi 2·1 promoter/luciferase construct cotransfected together with the wild-type GH receptor or various tyrosine-mutated GH receptors. As can be seen in Fig. 1, any one of the three tyrosines at positions 534, 566 and 627 was able to function in GH receptor transcriptional signaling when present as the only intracellular tyrosine.

As the transcription factor Stat5 has been implicated in the transcriptional activation by the GH receptor we investigated the ability of single tyrosine-containing mutant GH receptors to mediate GH-induced Stat5 DNA-binding activity in individual clones (Fig. 2A) or pools (Fig. 2B) of CHO cells transfected with various GH receptor tyrosine mutants. All clones expressed between 10 000 and 15 000 GH receptors per cell. In cells expressing GH receptors containing tyrosine 534, 566 or 627, a GH-induced band could be observed in gel-shift experiments using an Spi 2·1 promoter probe. The presence of Stat5 in the GH-induced complex is indicated by the ability of a Stat5 antibody to supershift this complex. In contrast, no DNA-binding activity was observed in cells expressing the tyrosine-deficient GH receptor
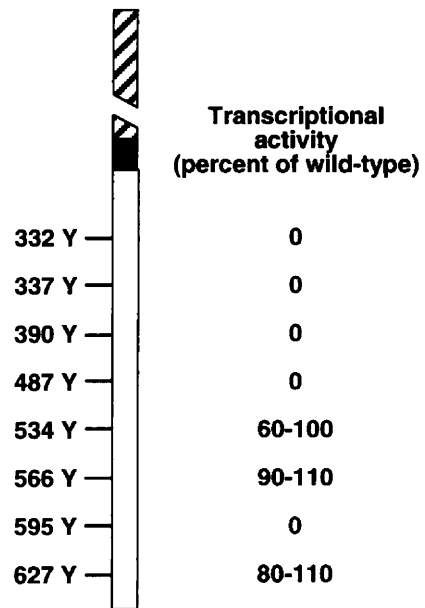

	Transcriptional activity (percent of wild-type)
332 Y —	0
337 Y —	0
390 Y —	0
487 Y —	0
534 Y —	60-100
566 Y —	90-110
595 Y —	0
627 Y —	80-110

FIGURE 1. The role of GH receptor intracellular tyrosines in transcriptional signaling. A schematic representaion of the porcine GH receptor is shown to the left, including the extracellular domain (hatched box), the transmembrane domain (solid box) and the intracellular domain (open box). The localization of the eight intracellular tyrosines is also indicated. The percent transcriptional activity refers to the ability of a GH receptor containing the indicated tyrosine as the only intracellular tyrosine to stimulate transcription of the Spi 2·1 promoter in a transient CAT (Hansen *et al.* 1996) assay (*n*=4) or luciferase assay (*n*=3) as described in Materials amd Methods. The numbers show the range of activitites observed compared with the transcriptional activation by the wild-type GH receptor.

mutant (mC8) or the GH receptor containing only tyrosine number 595 (mC8F595Y).

To further characterize the mechanism by which GH receptor tyrosine phosphorylation regulates Stat5 activation, we tested the ability of GH receptor-derived synthetic peptides to interfere with Stat5 binding to DNA. Activated Stat5 was isolated from the nuclei of GH-stimulated cells and incubated with synthetic peptides which were either non-phosphorylated or tyrosine phosphorylated. As shown in Fig. 3, peptides containing one of the phosphorylated tyrosines 534, 566 or 627 were able to inhibit binding of Stat5 to the Spi-GLE probe derived from the Spi 2·1 promoter dose-dependently. Although it appears from Fig. 3 that phosphopeptide 534 was less effective in inhibiting Stat5 DNA binding compared with phosphopeptides 566 and 627, this difference was not observed in subsequent experiments and thus probably
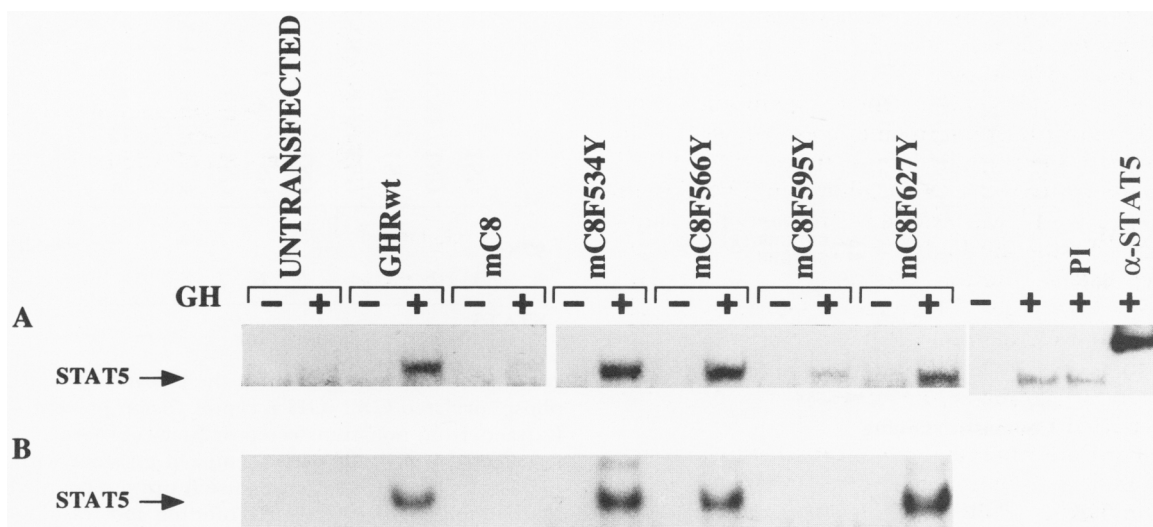
FIGURE 2. EMSA of GH-stimulated nuclear extracts from CHO cells expressing wild-type or tyrosine-mutated GH receptors. (A) Pools or (B) clones of GH receptor-transfected CHO cells were stimulated with 20 nM GH for 5 min and nuclear extracts were isolated. Binding of Stat5 to a ^{32}P-labeled Spi 2·1 promoter probe was determined as described in Materials and Methods. Nuclear extracts from GH-stimulated CHO cells expressing the wild-type GH receptor were incubated with preimmune serum (PI) or Stat5 antiserum (α-STAT5) as indicated. The results shown are representative of one of three independent experiments. GHRwt, wild-type GH receptor.
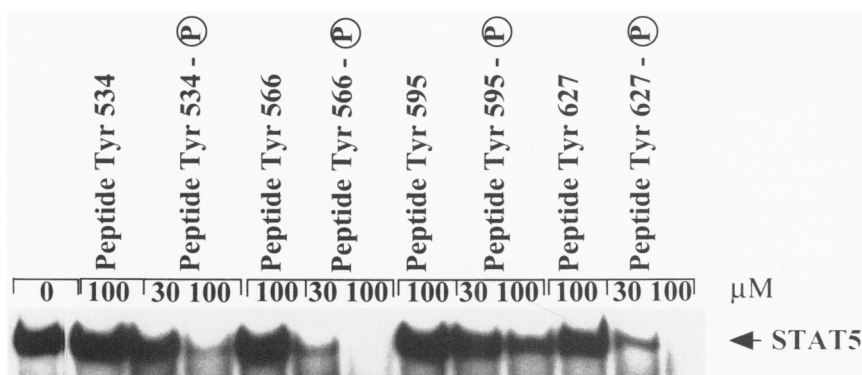

FIGURE 3. Inhibition of Stat5-DNA binding activity by phosphopeptides derived from the GH receptor. EMSA using nuclear extracts from GH-stimulated CHO cells expressing the wild-type GH receptor were performed in the presence of synthetic peptides or phosphopeptides derived from the GH receptor. Peptides at the indicated final concentrations were incubated together with the nuclear extract for 30 min at 4 °C prior to adding the DNA probe. The arrow indicates the migration of the Stat5/DNA complex. The results shown are representative of one of three independent experiments. P, phosphorylated.

represents experimental variance. None of the non-phosphorylated peptides affected Stat5 DNA binding. As a control, a peptide containing phosphotyrosine 595 did not affect Stat5 binding when tested at 30 μM and only slight inhibition was observed at 100 μM.

The inhibitory effects of GH receptor-derived peptides on Stat5 binding indicates a direct interaction between the Stat5 SH-2 domain with the phosphorylated GH receptor. Therefore in order to test this hypothesis we produced GST-GH receptor fusion proteins containing the C-terminal 184
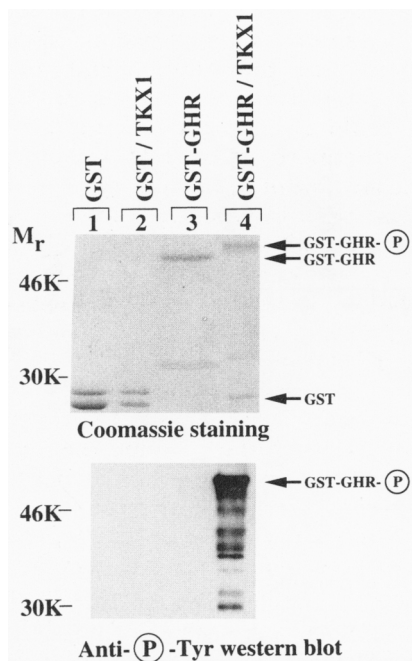
FIGURE 4.   Characterization of the GST-GH receptor fusion protein. Approximately 5 µg of the indicated protein was analyzed by SDS-PAGE followed by Coomassie blue staining (top panel) or 500 ng of the indicated protein by Western blotting using anti-phosphotyrosine antibodies (bottom panel). The arrows indicate the mobility of the non-phosphorylated GST-GH receptor (GST-GHR) or the tyrosine phosphorylated GST-GH receptor (GST-GHR-P). The results shown are representative of one of three independent experiments.
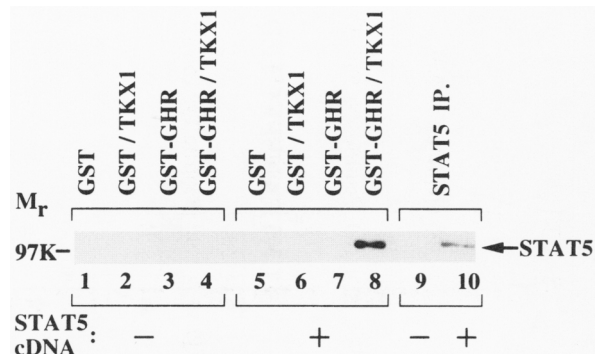

FIGURE 5.   Binding of Stat5 to the tyrosine phosphorylated GST-GH receptor fusion protein. Extracts from non-transfected or Stat5 cDNA-transfected COS cells were incubated together with the indicated GST-GH receptor fusion protein coupled to gluthathione Sepharose. After binding and washing, the bound proteins were analyzed by SDS-PAGE followed by Western blotting using a Stat5 antibody. Lanes 9 and 10 contain Stat5 immunoprecipitated (IP) material from non-transfected or Stat5-transfected COS cells respectively. The arrow indicates the mobility of Stat5. The results shown are representative of one of three independent experiments.

amino acids of the GH receptor. The fusion proteins were produced either in normal *E. coli* or in bacteria containing an inducible tyrosine kinase (TKX1 cells). The GST-GH receptor fusion proteins were analyzed by SDS-PAGE followed by Coomassie staining as shown in Fig. 4. The GST-GH receptor fusion protein produced by normal *E. coli* had an electrophoretic mobility according to the expected molecular mass of 52 kDa, whereas the tyrosine phosphorylated fusion protein produced in TKX1 cells had a reduced electrophoretic mobility which is characteristic of phosphorylated proteins (Fig. 4, lanes 3 and 4). Western blotting using anti-phosphotyrosine antibodies resulted in a strong signal which was present only in the lane containing GST-GH receptor protein from cells expressing the inducible tyrosine kinase. The GST protein itself was not tyrosine phosphorylated as indicated by the lack of signal in lane 2, and the identical mobility of the GST proteins from normal and TKX1 cells. It has not

been possible to determine which of the six tyrosine residues in the GH receptor (454–638) part of the fusion protein are phosphorylated. However, using mass spectrometry we have found that at least three of the tyrosines are phosphorylated (J A H and N B, data not shown). When the GST fusion proteins were incubated together with extracts from COS cells, Stat5 binding was observed only when the GST-GH receptor was tyrosine phosphorylated and only when the extracts were made from Stat5-transfected COS cells (Fig. 5). The Stat5 which was bound to the phosphorylated GST-GH receptor co-migrated with Stat5 immunoprecipitated from transfected COS cells (Fig. 5, lanes 8 and 10). Incubation of the GST-GH receptor together with the various GH receptor-derived synthetic peptides showed that the three peptides containing any of the tyrosines 534, 566 or 627 inhibited binding of Stat5 to the phosphorylated GST-GH receptor protein (Fig. 6). However, the peptide containing tyrosine 595 did not inhibit binding nor did any of the non-phosphorylated peptides.

DISCUSSION

Using transient co-transfection of CHO cells with a GH response element/luciferase construct together with mutated GH receptors in which all but one of
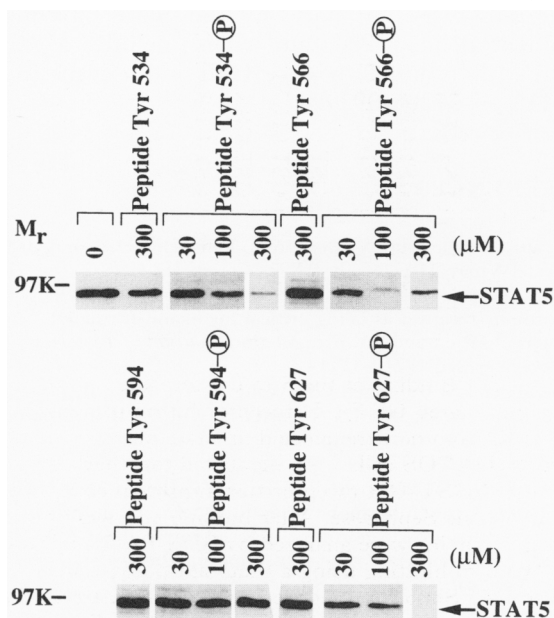
FIGURE 6. Inhibition of Stat5 binding to the GH receptor by phosphopeptides derived from the GH receptor. Cell extracts from Stat5-transfected COS cells were incubated with tyrosine phosphorylated GST-GH receptor fusion protein in the presence of the peptide or phosphopeptide at the indicated concentrations. After binding and washing, the bound proteins were analyzed by SDS-PAGE followed by Western blot analysis using a Stat5 antibody. The arrows indicate the mobility of Stat5. The results shown are representative of one of three independent experiments.

the intracellular tyrosines were mutated to phenylalanines, we identified specific tyrosines (at positions 534, 566 and 627) required for transcriptional signaling; this is in agreement with previous studies (Hansen *et al.* 1996). However, in the present study, we used a luciferase reporter gene which allows us to detect GH induction after only 6 h of stimulation, in contrast to 48 h needed for CAT activity. The shorter stimulation time further supports a direct involvement of GH on transcription, in contrast to possible long-term effects of GH on post-transcriptional events such as mRNA stabilization or increased translational activity.

We (Hansen *et al.* 1996) and others (Wang *et al.* 1995*b*) have recently shown that GH receptor tyrosines are not required for GH stimulation of Jak2 activity, MAP kinase activity, mitogenic signaling, Stat1 activation or Ca^{2+} signaling. However, the activation of Stat5 was abolished in cells expressing a GH receptor lacking intracellular tyrosines. By replacing individual tyrosines into the tyrosine-deficient GH receptor we found three

tyrosines which, when present as the only intracellular tyrosines, could function in mediating GH-induced gene transcription and that each of these three tyrosines involved in transcriptional signaling could also function in the activation of Stat5. Furthermore, these GH receptor mutants containing any one of these three tyrosines were just as active as the wild-type GH receptor, indicating redundancy in this portion of the GH receptor. Interestingly, it was recently reported that the three GH receptor tyrosine residues 534, 566 and 627 are phosphorylated in response to GH in transfected mouse L-cells (Wang *et al.* 1996).

In other receptors of the cytokine receptor superfamily, specific tyrosines have been implicated in signaling. In both the LIF and the interleukin 4 (IL-4) receptors as well as in the signal transducer gp13O, specific tyrosines were found to be required for Stat activation (Hou *et al.* 1994, Stahl *et al.* 1995). The activation of Stat factors by activated cytokine receptors probably involves interaction of the SH-2 domain of the Stat factors with a phosphotyrosine in the receptor. This concept is supported by the finding that the amino acid sequences C-terminal to the tyrosine residues found to be important for IL-4 and LIF receptor signaling are homologous, since the specificity of SH-2 domain binding to phosphotyrosines is determined by the amino acids located in the +1 to +3 positions relative to the phosphotyrosine (Songyang *et al.* 1993). Surprisingly, the amino acid sequence C-terminal to the three tyrosines of the GH receptor found to be involved in signaling showed no obvious similarity (534-YFCEA, 566-YITTE and 627-YVSTD) other than a hydrophobic residue at position +1. However, phosphopeptides containing one of these three sequences were able to interfere with Stat5 DNA binding and to compete for Stat5 binding to the phosphorylated GST-GH receptor fusion protein. This observation suggests that the SH-2 domain of Stat5 is able to bind phosphotyrosines, having quite different amino acids at the +1 and +3 positions. Taken together, these results strongly support the two-step model of Stat activation by cytokine receptors proposed by Stahl *et al.* (1995) and Hou *et al.* (1994), suggesting that the first step in Stat activation is specific tyrosine phosphorylation of the receptor and that this phosphotyrosine serves as a binding site for the latent cytoplasmic form of the Stat factor. However, from our studies it cannot be excluded that other SH-2 domain-containing factors are present and that Stat5 binds to the GH receptor via such adaptor proteins. Following receptor binding, the Stat factor becomes tyrosine phosphorylated and forms a dimer by intermolecular SH-2 domain

phosphotyrosine binding. The Stat dimer then translocates to the nucleus where it binds specific GLE sequences in the promoters of cytokine-responsive genes.

Several other GH receptor-mediated signaling pathways do not require GH receptor tyrosine phosphorylation. The activation of voltage-dependent Ca^{2+} channels by GH was not affected in cells expressing GH receptors in which all intracellular tyrosines were mutated to phenylalanine (Hansen *et al*. 1996). This is in agreement with the finding that GH-induced Ca^{2+} signaling is independent of tyrosine kinase activity (Ilondo *et al*. 1994). GH stimulation of cell proliferation, Stat1 activation and MAP kinase activity was also normal in cells expressing GH receptors lacking intracellular tyrosines (Wang *et al*. 1995*b*, Hansen *et al*. 1996), despite the fact that these pathways are dependent upon Jak2 activation and tyrosine phosphorylation, suggesting that other Jak2 kinase substrates must exist which act as intermediates between the activated tyrosine kinase and downstream signaling. Since the Jak2 kinase itself is tyrosine phosphorylated and since a chimeric GH receptor in which the entire intracellular domain was replaced by Jak2 could mediate GH stimulation of Stat3 activation (Yi *et al*. 1996), it may be speculated that phosphotyrosines on Jak2 itself mediate activation of Stat3.

In conclusion, we have shown that three tyrosine residues of the GH receptor are involved in transcriptional signaling by serving as direct binding sites for Stat5 in their phosphorylated state. This is in contrast to GH activation of Stat3 which does not require GH receptor tyrosine phosphorylation. In addition, in view of the fact that we have previously identified different tyrosine residues in the GH receptor which are required for metabolic signaling pathways, it appears that GH can activate several independent signal transduction pathways which are mediated by discrete domains of the intracellular portion of its receptor.

ACKNOWLEDGEMENTS

REFERENCES

Allevato G, Billestrup N, Goujon L, Petersen ED, Norstedt G, Postel-Vinay M-C, Kelly PA & Nielsen JH 1995 Identification of phenylalanine 346 in the rat growth hormone receptor as being critical for ligand-mediated internalization and down-regulation. *Journal of Biological Chemistry* **270** 17210–17214.

Argetsinger LS, Campbell GS, Wang X, Witthuhn BA, Silvennoinen O, Ihle JN & Carter-Su C 1993 Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell* **74** 237–244.

Billestrup N, Bouchelouche P, Allevato G, Ilondo M & Nielsen JH 1995 Growth hormone receptor C-terminal domains required for growth hormone-induced intracellular free Ca^{2+} oscillations and gene-transcription. *Proceedings of the National Academy of Sciences of the USA* **92** 2725–2729.

Campbell GS, Pang L, Miyasaka T, Saltiel AR & Carter-Su C 1992 Stimulation by growth hormone of MAP kinase activity in 3T3-F442A fibroblasts. *Journal of Biological Chemistry* **267** 6074–6080.

Campbell GS, Meyer DJ, Raz R, Levy DE, Schwartz J & Carter-Su C 1995 Activation of acute phase response factor (APRF)/stat3 transcription factor by growth hormone. *Journal of Biological Chemistry* **270** 3974–3979.

Carter-Su C, Stubbart JR, Wang X, Stred SE, Argetsinger LS & Shafer JA 1989 Phosphorylation of highly purified growth hormone receptors by a growth hormone receptor-associated tyrosine kinase. *Journal of Biological Chemistry* **264** 18654–18661.

Gouilleux F, Pallard C, Dusanter-Fourt I, Wakao H, Haldosen L-A, Norstedt G, Levy & Groner B 1995 Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. *EMBO Journal* **14** 2005–2013.

Goujon L, Allevato G, Simonin G, Paquereau L, LeCam A, Clarks J, Nielsen JH, Djiane J-M, Postel-Vinay M-C, Edery M & Kelly PA 1994 Cytoplasmic sequences of growth hormone receptor necessary for signal transduction. *Proceedings of the National Academy of Sciences of the USA* **91** 957–961.

Hansen LH, Wang X, Kopchick JJ, Bouchelouche P, Nielsen JH, Galsgaard ED & Billestrup N 1996 Identification of tyrosine residues in the intracellular domain of the growth hormone receptor required for transcriptional signaling and stat5 activation. *Journal of Biological Chemistry* **271** 12669–12673.

Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M & McKnight SL 1994 An interleukin-4-induced transcription factor: IL-4 Stat. *Journal of Biological Chemistry* **265** 1701–1706.

Ilondo MM, DeMeyts P & Bouchelouche P 1994 Human growth hormone increases cytosolic free calcium in cultured human IM-9 lymphocytes: a novel mechanism of growth hormone transmembrane signalling. *Biochemical and Biophysical Research Communications* **202** 391–397.

Kelly PA, Djiane J, Postel-Vinay M-C & Edery M 1991 The prolactin/growth hormone receptor family. *Endocrine Reviews* **12** 235–251.

Lobie PE, Allevato G, Nielsen JH, Norstedt G & Billestrup N 1995 Requirement of tyrosine residues 333 and 338 of the growth hormone (GH) receptor for selected GH-stimulated function. *Journal of Biological Chemistry* **270** 21745–21750.

Mathews LS, Enberg B & Norstedt G 1989 Regulation of rat growth hormone receptor gene expression. *Journal of Biological Chemistry* **264** 9905–9910.

Møldrup A, Allevato G, Dyrberg T, Nielsen JH & Billestrup N 1991 Growth hormone action in rat insulinoma cells expressing truncated growth hormone receptors. *Journal of Biological Chemistry* **266** 17441–17445.

Moller C, Hansson A, Enberg B, Lobie PE & Norstedt G 1992 Growth hormone induction of tyrosine phosphorylation and activation of mitogen activated protein kinases in cells transfected with rat GH receptor cDNA. *Journal of Biological Chemistry* **267** 23403–23408.

Ram PA, Park S, Choi HK & Waxman DJ 1996 Growth hormone activation of Stat 1, Stat 3 and Stat 5 in rat liver. *Journal of Biological Chemistry* **271** 5929–5940.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou M, Hanafusa H, Schaffhausen B & Cantley LC 1993 SH2 domains recognize specific phosphopeptide sequences. *Cell* **72** 767–778.

Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE & Yancopoulos GD 1995 Choice of STAT's and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* **267** 1349–1353.

VanderKuur J A, Wang X, Zhang L, Campbell GS, Allevato G, Billestrup N, Norstedt G & Carter-Su C 1994 Domains of the growth hormone receptor required for association and activation of JAK2 tyrosine kinase. *Journal of Biological Chemistry* **269** 21709–21717.

VanderKuur J, Billestrup N, Allevato A, Norstedt G & Carter-Su C 1995a Growth hormone promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb-2. *Journal of Biological Chemistry* **270** 7587–7593.

VanderKuur J, Wang X, Zhang L, Allevato G, Billestrup N & Carter-Su C 1995b GH dependent phosphorylation of tyrosine 333 and/or 338 of the growth hormone receptor. *Journal of Biological Chemistry* **270** 21738–21744.

Wang X, Souza SC, Kelder B, Cioffi JA & Kopchick JJ 1995a A 40-amino acid segment of the growth hormone receptor cytoplasmic domain is essential for GH induced tyrosine phosphorylated cytosolic proteins. *Journal of Biological Chemistry* **270** 6261–6266.

Wang X, Darus CJ, Xu BC & Kopchick JJ 1996 Identification of growth hormone receptor (GHR) tyrosine residues required for GHR phosphorylation and JAK2 and STAT5 activation. *Molecular Endocrinology* **10** 1249–1260.

Wang Y-D, Wong K & Wood WI 1995b Intracellular tyrosine residues of the human growth hormone receptor are not required for the signaling of proliferation or jak-stat activation. *Journal of Biological Chemistry* **270** 7021–7024.

Yi W, Kim SO, Jiang J, Park SH, Kraft AS, Waxman DJ & Frank SJ 1996 Growth hormone receptor cytoplasmic domain differentially promotes stat3 and stat5b tyrosine phosphorylation by jak2 kinase. *10th International Congress of Endocrinology, San Francisco, 12–15 June.*