Requirement of Tyrosine Residues 333 and 338 of the Growth Hormone (GH) Receptor for Selected GH-stimulated Function*

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We have examined the involvement of tyrosine residues 333 and 338 of the growth hormone (GH) receptor in the cellular response to GH. Stable Chinese hamster ovary (CHO) cell clones expressing a receptor with tyrosine residues at position 333 and 338 of the receptor substituted for phenylalanine (CHO-GHR1–638Y333F, Y338F) were generated by cDNA transfection. Compared with the wild type receptor the Y333F, Y338F mutant possessed normal high affinity ligand binding, hormone internalization, and ligand-induced receptor down-regulation. GH activation of mitogen-associated protein kinase was also similar in CHO clones expressing similar wild type and Y333F, Y338F receptor number. However, two GH-regulated cellular events (lipogenesis, and protein synthesis) were deficient in the tyrosine substituted receptor. In contrast, transcriptional regulation by GH (as evidenced by chloramphenicol acetyltransferase cDNA expression driven by the GH-responsive region of the SPI 2.1 gene) was not affected by Y333F, Y338F substitution. Thus we provide the first experimental evidence that specific tyrosine residues of the GH receptor are required for selected cellular responses to GH.

The GH1 receptor is a member of the cytokine receptor superfamily (Bazan, 1990; Cosman et al., 1990; Kitamura et al., 1994). One characteristic of this family of receptors is the lack of a canonical sequence for intrinsic tyrosine kinase activity (Leung et al., 1987; Mathews et al., 1989; Smith et al., 1989; Kitamura et al., 1994). However, ligand binding to a number of these receptors, including the GH receptor, induces tyrosyl phosphorylation of cellular proteins (Möller et al., 1992; Wang et al., 1992, 1993). This tyrosyl phosphorylation of cellular proteins is attributed to the association with Janus kinase 2 (JAK2) for the GH receptor (Argetsinger et al., 1993) and also the receptors for erythropoietin, prolactin, interleukin-3, interleukin-6, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, leukocyte inhibitory factor, oncostatin M, and ciliary neurotrophic factor (Silvennoinen et al., 1993; Stahl et al., 1994; Wittthuhn et al., 1993; Narazaki et al., 1994; Dusant-Fourt et al., 1994; DaSilva et al., 1994). Pharmacological inhibition of cellular tyrosine phosphorylation does inhibit certain GH-stimulated cellular events (Campbell et al., 1993; Möller et al., 1994; Sliva et al., 1994). Although the GH receptor is itself tyrosine-phosphorylated (Foster et al., 1988), it has not been demonstrated that such receptor phosphorylation is required for the biological response to GH.

Studies involving truncation of the GH receptor intracellular domain has suggested functional specificity for different regions of the intracellular domain. Thus the membrane proximal portion alone (truncated at amino acid 454) is capable of mediation of GH-stimulated mitogenesis (Möller et al., 1992; Cotter et al., 1993), lipid synthesis (Möller et al., 1994), protein synthesis (Billestrup et al., 1994), MAP kinase activation (Möller et al., 1992), c-fos expression (VanderKuur et al., 1994) and hormone internalization (Möldrup et al., 1991). In contrast the carboxyl-terminal portion of the GH receptor is required for full transcriptional activation (Goujon et al., 1994) and phosphorylation of p97 (a putative GH-dependent transcription factor) (VanderKuur et al., 1994). The membrane proximal portion of the intracellular domain contains 4 tyrosine residues but a single set of paired tyrosine residues at position 333 and 338 of the receptor. Phosphorylation of specific tyrosyl residues in receptor molecules serve as docking sites for one or more proteins containing SH2 domains (Koch et al., 1991; Cantley et al., 1991; Mayer and Baltimore, 1993; Nishimura et al., 1993). Concordant with functional specificity of other receptor molecules being determined by phosphorylation of specific tyrosine residues (Mohammadi et al., 1992; Pawson and Gish, 1992), we have investigated the importance of tyrosine 333 and 338 of the GH receptor for the biologic response to GH. We show here that phosphorylation of tyrosine 333 and 338 are required for selected biological functions of GH.

EXPERIMENTAL PROCEDURES

Materials—Recombinant hGH was a generous gift of both Pharmacia (Stockholm, Sweden) and Novo-Nordisk. Materials for cell culture were obtained from Life Technologies, Inc. [3H]Glucose, [14C]chloramphenicol, and [125I] were obtained from Amersham (Amer- sham, United Kingdom). Triton X-100, dexamethasone, myelin basic protein, and BSA were from Sigma. The [14C]chloramphenicol acetyltransferase expression plasmid (pCH110) was obtained from Pharmacia (Uppsala, Sweden). MAP kinase monoclonal antibody was purchased from Zymed Laboratories, Inc. (S. San Francisco, CA). All other reagents were of reagent grade or higher.

Cell Culture—CHO cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum, 50 µg/ml penicillin, and 50 µg/ml streptomycin (Möller et al., 1992).

Cellular Transfection—Rat GH receptor cDNA was cloned into an expression plasmid containing an SV40 enhancer and a human metallothionein IIa promoter. The cDNAs were transfected into CHO-K1 cells with Lipofectin together with the pPB-1 plasmid which contains a neomycin resistance gene fused to the thymidine kinase promoter. Stable integrants were selected using 1000 µg/ml G418. The complete rat GH receptor cDNA (Mathews et al., 1989) coding for amino acids 1–638 was expressed in CHO4–638 (Emtner et al., 1990) or CHO-A-638 cells (CHO-GHR1–638) (Wang et al., 1993). A stop codon was created at amino acid 295 by in vitro mutagenesis of GH receptor cDNA to create a membrane-bound, but cytoplasmic domain-deficient, receptor. This

* This work was funded in part by a grant from Pharmacia AB (Stockholm, Sweden). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: GH, growth hormone; MAP, mitogen-associated protein; BSA, bovine serum albumin; hGH, human growth factor; CAT, chloramphenicol acetyltransferase.

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cDNA was expressed in CHO-294 (CHO-GHR<sub>1–638</sub>) cells (Möller et al., 1992). The Y333F,Y338F mutant cDNA was constructed using polymerase chain reaction as described. Oligonucleotides carrying the substitution were synthesized and used as primers in order to introduce point mutations. The introduced mutation was confirmed by DNA sequence analysis. This cDNA was expressed in CHO-GHR<sub>1–638</sub> Y333F,Y338F cells (VanderKuur et al., 1995b).

**Hormone Internalization, Degradation, and Receptor Turnover—** CHO cells were plated in six-well plates (Moldrup et al., 1991). Cell monolayers were washed in HEPES binding buffer (10 mM HEPES, pH 7.4, 124 mM NaCl, 4 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>) and incubated on ice for 3 h in 1 ml of binding buffer containing 100,000 cpm [<sup>125</sup>I]-hGH. The medium was removed, replaced with HEPES binding buffer, and cells were washed three times in wash buffer containing 10 mM HEPES, pH 7.4, 124 mM NaCl, 4 mM CaCl<sub>2</sub>, and 1.5 mM MgCl<sub>2</sub>. The cells were then incubated for 90 min at room temperature in binding medium (10 mM HEPES, pH 7.4, 1% human serum albumin) containing [<sup>125</sup>I]-hGH (approximately 100,000 cpm). The cells were washed four times in wash buffer and solubilized with 1 ml of 1 N NaOH. Radioactivity was determined by precipitation in 10% trichloroacetic acid and quantitation of radioactivity in the pellet and supernatant.

For estimation of ligand-induced receptor down-regulation, cell monolayers in six-well plates were incubated in the presence of different concentrations of hGH in serum-free medium for 12 h (Roupas and Herington, 1988). The cells were placed on ice and associated hGH was removed by acid wash (0.15 M NaCl, 0.05 M glycine, pH 2.5) and further washed three times in a wash buffer containing 10 mM HEPES, pH 7.4, 124 mM NaCl, 4 mM CaCl<sub>2</sub>, and 1.5 mM MgCl<sub>2</sub>. The cells were then incubated for 90 min at room temperature in binding medium (10 mM HEPES, pH 7.4, 1% human serum albumin) containing [<sup>125</sup>I]-hGH (approximately 100,000 cpm). The cells were washed four times in wash buffer and solubilized with 1 ml of 1 N NaOH. Radioactivity was determined by precipitation in 10% trichloroacetic acid and quantitation of radioactivity in the pellet and supernatant.

**MAP Kinase Activity—** Cells were cultured in 100-mm dishes to near confluence and serum-deprived for 12 h before stimulation with 20 ng/ml of hGH. The MAP kinase activity was measured in vivo. CAT assays were performed as described elsewhere. The introduction of the mutant was confirmed by DNA sequence analysis. This cDNA was expressed in CHO-GHR<sub>1–638</sub> Y333F,Y338F cells (VanderKuur et al., 1995b).

**Lipid Synthesis—** Lipogenesis was estimated as described by Moldy et al. (1974) with minor modifications. Cells were grown to confluence in six-well plates and incubated in serum-free medium (Ham’s F-12) supplemented with 1% (v/v) BSA for 12 h. The assay was initiated by the addition of 1 ml of Krebs-Ringer-HEPES buffer containing 1% (w/v) BSA, 0.55 mM glucose, 0.05 mM glycine, pH 2.5) and further washed three times in a wash buffer containing 10 mM HEPES, pH 7.4, 124 mM NaCl, 4 mM CaCl<sub>2</sub>, and 1.5 mM MgCl<sub>2</sub>. The cells were then incubated for 90 min at room temperature in binding medium (10 mM HEPES, pH 7.4, 1% human serum albumin) containing [<sup>125</sup>I]-hGH (approximately 100,000 cpm). The cells were washed four times in wash buffer and solubilized with 1 ml of 1 N NaOH. Radioactivity was counted in a gamma counter. Nonspecific binding was estimated in the presence of 1 μg/ml unlabeled hGH and was <5%.

**Protein Synthesis—** Cells were grown to confluence in 60-mm dishes and washed with phosphate-buffered saline, and incubated in serum-free medium (Ham’s F-12) supplemented with 1% (w/v) BSA for 12 h. The assay was initiated by the addition of 1 ml of Krebs-Ringer-HEPES buffer containing 1% (w/v) BSA, 0.55 mM glucose, 0.05 μCi of [¹⁴C]leucine (5–15 Ci/mmol) ± hGH (100 nM). The incubation was continued for 2 h and terminated by washing with cold phosphate-buffered saline followed by the addition of 0.5 ml of 0.5 N NaOH, 0.1% (v/v) Triton X-100. Solubilized cells were transferred to scintillation vials containing 3.5 ml of a toluene scintillant, and lipid-incorporated radioactivity was measured in the organic phase by scintillation counting. Results are expressed as the percentage induction above control, where cells were incubated in the absence of hGH.

**RESULTS**

Characterization of Clones Stably Transfected with cDNA for the GH Receptor—CHO cells stably expressing the full-length receptor have been described previously (Möller et al., 1992; Wang et al., 1993). The generation and characterization of stable clones expressing the Y333F,Y338F mutation (CHO-GHR<sub>1–638</sub>Y333F,Y338F) and for the Y333F,Y338F receptor (GHR<sub>1–638</sub>Y333F,Y338F) was quantified by liquid scintillation counting. Results are presented as the fold-stimulation above cells assayed in the absence of hGH.

Transient Transfection of SPI-CAT Reporter Constructs—Cells were grown to 50% confluence in 60-mm dishes. Twenty-four hours prior to transfection, the cells were washed twice with Dulbecco’s modified Eagle’s medium and serum-free medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 supplemented with 10 μg/ml of transferrin, 80 μg/ml insulin, 2 mg/ml glucose, and nonessential amino acids. CHO cells were transiently transfected by the calcium phosphate procedure with 3 μg of pCH110 (β-galactosidase expression vector, Pharmacia, Uppsala, Sweden), 1.5 μg of the construct containing the bacterial chloramphenicol acetyltransferase (CAT) coding sequence linked to -149 to +103 of the serum protease inhibitor (SPI 2.1) gene promoter (Yoon et al., 1990) and 3 μg of the Y333F,Y338F GH receptor or wild type receptor plasmids. After the cells were subjected to glycerol shock, fresh GC3 medium with dexamethasone (10 μM) was added ± hGH. Cells were scraped from the plate after 48 h, and extracts were prepared by three consecutive freeze-thaw cycles followed by centrifugation at 15,000 × g for 10 min. Aliquots of the supernatant were normalized for β-galactosidase activity as assayed for CAT activity. CAT assays were performed as described with 1 μCi of [¹⁴C]chloramphenicol. The samples were subjected to thin layer chromatography and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). CAT activity was determined by quantification of the spots corresponding to [¹⁴C]chloramphenicol and its acetylated forms.
Y333F, Y338F substitution of the receptor (Moeller et al., 1992; VanderKuur et al., 1992). GH has also been demonstrated previously to stimulate lipogenesis in GH receptor cDNA-transfected cells (Moller et al., 1994). This effect also does not require the carboxy-terminal half of the receptor intracellular domain. We therefore examined GH-stimulated lipogenesis in the different CHO stable transfectants. GH treatment of CHO-GHR1–638 cells resulted in a 2-fold increase in cellular lipogenesis within 2 h after addition of hormone. Neither of the CHO-GHR1–638, Y333F, Y338F clones nor CHO-GHR1–294 cells displayed any significant enhancement of protein synthesis upon GH stimulation (Fig. 5).

Lipogenesis in CHO-GHR1–638Y333F, Y338F—GH has been demonstrated previously to stimulate lipogenesis in GH receptor cDNA-transfected cells (Moller et al., 1994). This effect also does not require the carboxy-terminal half of the receptor intracellular domain. We therefore examined GH-stimulated lipogenesis in the different CHO stable transfectants. GH treatment of CHO-GHR1–638 cells resulted in a 2-fold increase in cellular lipogenesis within 2 h after addition of hormone. Neither of the CHO-GHR1–638, Y333F, Y338F clones nor CHO-GHR1–294 cells displayed any significant enhancement of protein synthesis upon GH stimulation (Fig. 5).

SPI-CAT Expression in CHO-GHR1–638Y333F, Y338F—GH has been demonstrated previously to drive CAT cDNA expression in reporter constructs containing multiple repeats of a 45-base pair GH-responsive region of the SPI 2.1 gene promoter (Yoon et al., 1990; Enberg et al., 1994; Goujon et al., 1994; Silva et al., 1994). These transcriptional regulatory effects of GH require the entire intracellular domain (Enberg et al., 1994; Goujon et al., 1994; Silva et al., 1994). This is in contrast to GH-stimulated lipogenesis, protein synthesis, mitogenesis, and MAP kinase activation which require only the membrane proximal portion of the intracellular domain. We therefore examined the ability of GH to stimulate transcription via the Y333F, Y338F receptor. As is evident from Fig. 6 both the wild type receptor and the Y333F, Y338F receptor drive CAT expression to a similar extent. Thus Tyr333 and Tyr338 of the GH receptor are not involved in GH regulation of transcription.
DISCUSSION

We demonstrate here that specific tyrosine residues within the GH receptor are required for GH to elicit selective cellular function. We refer specifically to Tyr\(^{333}\) and Tyr\(^{338}\) of the GH receptor and their involvement in GH-mediated protein synthesis and lipogenesis. The selective retention of certain GH-stimulated functions within the cell (also see VanderKuur et al., 1995b) further substantiates the specific involvement of these residues in GH signal transduction.

Several studies have implicated the existence of distinct receptor domains regulating GH-stimulated events (Moldrup et al., 1991; Möller et al., 1992, 1994; Colosi et al., 1993; Enberg et al., 1994; Goujon et al., 1994; VanderKuur et al., 1994). Truncation of the intracellular domain of the receptor has allowed definition of two distinct macrodomains within the receptor with differential involvement in transcription and metabolic events. A complete transcriptional response to GH requires the presence of the carboxyl-terminal half of the intracellular domain. In contrast certain presumed “metabolic” events such as mitogenesis, lipogenesis and protein synthesis are mediated by the membrane proximal portion of the intracellular domain. That specific tyrosine residues are necessary for selected functions further reinforces the concept of distinct receptor domains. However, since no absolute delineation exists (e.g. both MAP kinase activation and protein synthesis require only the membrane-proximal portion of the intracellular domain, yet only protein synthesis is deficient in the Y\(^{333}\)F, Y\(^{338}\)F receptor), then regional functional specificity of the receptor is likely to be dictated by the association of specific signaling molecules to discrete phosphorylated tyrosine residues. Such is the case for the better characterized epidermal growth factor and platelet-derived growth factor receptors, where specific phosphorylated tyrosine residues serve as docking sites for proteins containing SH2 domains (Nishimura et al., 1993). That transcriptional up-regulation is not affected by Y\(^{333}\)F, Y\(^{338}\)F substitution in the receptor indicates that some more distal

FIG. 4. GH stimulation of protein synthesis in CHO cells stably transfected with cDNA for the wild type GH receptor (GHR\(_{1–638}\)), a receptor expressing only 5 amino acids in the intracellular domain (GHR\(_{1–294}\)), and receptors in which tyrosines 333 and 338 have been substituted with phenylalanine (GHR\(_{1–638}\) Y\(^{333}\)F, Y\(^{338}\)F (clones 3 and 23)). Confluent cells were incubated in serum-free medium for 24 h before addition of 100 nM hGH for 12 h. Protein synthesis was estimated by the incorporation of \(_{3}^{[}\text{H}\) leucine during a 2-h pulse-chase. \(_{3}^{[}\text{H}\) Leucine incorporated into proteins was precipitated by trichloroacetic acid and collected on glass fiber filters. Radioactivity was estimated by scintillation counting. Results are presented as mean ± S.D. from triplicate determinations of the fold stimulation above untreated cells. Results for GHR\(_{1–638}\) presented here were derived from CHO4 cells with similar results being obtained with CHO4A cells. 

FIG. 5. GH stimulation of lipid synthesis in CHO cells stably transfected with cDNA for the wild type GH receptor (GHR\(_{1–638}\)), a receptor expressing only 5 amino acids in the intracellular domain (GHR\(_{1–294}\)) and receptors in which tyrosines 333 and 338 have been substituted with phenylalanine (GHR\(_{1–638}\) Y\(^{333}\)F, Y\(^{338}\)F (clones 3 and 23)). Confluent cells were incubated in serum-free medium containing 1% BSA for 24 h before commencement of assay. Lipogenesis was estimated by the incorporation of \(_{3}^{[}\text{H}\) glucose into the lipid-soluble fraction of the cell. Radioactivity was estimated by scintillation counting. Results are presented as mean ± S.D. from triplicate determinations of the fold stimulation (in the presence of 100 nM hGH) above untreated cells. Results presented here for GHR\(_{1–638}\) are derived from CHO4 cells.

FIG. 6. GH stimulation of CAT activity driven by the SPI-GH-responsive element in untransfected CHO cells, in CHO cells transiently transfected with cDNA for the wild type GH receptor (GHR\(_{1–638}\)), and in CHO cells transiently transfected with cDNA for the Y\(^{333}\)F, Y\(^{338}\)F receptor (GHR\(_{1–638}\) Y\(^{333}\)F, Y\(^{338}\)F). CHO cells were transiently transfected by the calcium phosphate procedure and also with a construct containing bacterial CAT coding sequence linked to −149/−103 of the serine protease inhibitor (SPI 2.1) gene promoter. The cells were processed, and CAT assays were performed as described under “Experimental Procedures.” Results are mean ± S.D. from triplicate determinations.
tyrosine residue(s) may be involved in this event. GH has recently been demonstrated to activate transcription by phosphorylation of a factor binding to a GAS-like DNA element (Meyer et al., 1994; Finbloom et al., 1994; Silva et al., 1994). It has been reported that this factor is p91 (STAT1) and STAT3-like for the SIE element (Meyer et al., 1994; Finbloom et al., 1994; Campbell et al., 1995) and STAT5-like for the SPI 2.1 GAS-like element (Wood et al., 1995). The γ-interferon receptor requires a distal tyrosine residue (Tyr\(^{440}\)) for activation of STAT 1 and presumably functions through direct receptor-STAT1 interaction (Greenlund et al., 1994). The GH receptor could also hypothetically participate in transcriptional activation by providing a distal tyrosine residue for docking and subsequent phosphorylation of the STAT protein. Also receptor dimerization (Cunningham et al., 1991; Devos et al., 1992), presumably after ligand binding, could present a conformation that allows docking of signaling molecules regardless of the phosphorylation state. At present we do not know the nature of the molecular interaction(s) that is potentially disrupted by the Y333F, Y338F substitution. These tyrosines are not part of a known sequence reported to serve as docking sites for signaling molecules (Cantley et al., 1991).

The GH stimulation of protein synthesis does not require RNA synthesis (Martin and Young, 1965). Instead GH treatment of animals is associated with an apparent increase in ribosomal efficiency (Kostyo and Rilemma, 1971). Increase in cellular protein synthesis is thought to be mediated by phosphorylation of the ribosomal protein S6. The phosphorylation of ribosomal protein S6 is mediated by S6 kinase, which is itself phosphorylated by MAP kinase (Maller, 1991). GH has been reported to activate both MAP kinase (Winston and Bertics, 1991) and presumably functions through direct receptor-phosphorylation state. At present we do not know the nature of the molecular interaction(s) that is potentially disrupted by the Y333F, Y338F substitution. These tyrosines are not part of a known sequence reported to serve as docking sites for signaling molecules (Cantley et al., 1991).

It has been reported that this factor is p91 (STAT1)- and presumably functions through direct receptor-phosphorylation state. Atpresentwedonotknowthenatureof the knowledge reported to serve as docking sites for signaling molecules (Cantley et al., 1991).

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