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Biochemistry

Cytoplasmic sequences of the growth hormone receptor necessary for signal transduction

(growth hormone receptor mutants/transient transfection/serine protease inhibitor/ovine β-lactoglobulin/promoter tests)

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ABSTRACT **To study structure–function relationships of the growth hormone (GH) receptor (GHR), two functional systems have been developed. CHO cells were transiently cotransfected with the cDNA encoding the full-length rat GHR and with a construct consisting of the 5′ flanking region of one of two GH-dependent genes encoding ovine β-lactoglobulin or serine protease inhibitor 2.1 (Spi 2.1, formerly Spi.1; the corresponding rat gene has recently been redesignated *Spin2a*) coupled to the bacterial reporter gene encoding chloramphenicol acetyltransferase (CAT). Transfected cells were grown in the absence and presence of human GH and dexamethasone for the Spi 2.1 gene construct. GH was able to activate each promoter (with ≈4-fold induction of CAT activity) in a dose-dependent manner. For both tests, the maximal effect was observed at 20 nM human GH. These tests have been used to identify functional domains of the GHR. Two truncated (T) GHRs, lacking most or part of the cytoplasmic domain [called T276 (ending at residue 276) and T436 (ending at residue 436)], were unable to stimulate CAT activity. The GHR contains a proline-rich region, called "Box I," conserved in the cytokine/GH/prolactin receptor family. Alanine substitutions for the four prolines of GHR Box I were introduced. Single proline-to-alanine mutations did not affect the functional activity of the GHR. However, modification of the four prolines together or deletion of the Box I (15 amino acids between positions 279 and 293) resulted in the complete absence of GH stimulation. Thus, the proline-rich region, shown to be important for other members of this receptor superfamily, is also critical for GH signal transduction.**

The initial step of growth hormone (GH) action is the binding to a membrane receptor. The GH receptor (GHR) is a protein of 620 amino acids (aa) consisting of an extracellular hormone-binding domain of 246 aa, a single transmembrane region, and a cytoplasmic domain of 350 aa (1). The GHR belongs to the cytokine/GH/prolactin (PRL) receptor (PRLR) family (2). It contains no tyrosine-kinase or other known consensus motifs.

Site-directed mutagenesis has been successfully used for the identification of domains of functional importance in other growth factor receptors, but lack of suitable GH-responsive cell systems has hampered this approach for the GHR. Billestrup *et al.* (3) and Emtner *et al.* (4) have described bioassays for the GHR, but these tests require stable cell transfections, which renders the study of multiple mutations of the GHR difficult.

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To establish a bioassay based on specific gene activation by GH that would be suitable with transient cell transfections, we used promoters of genes encoding β-lactoglobulin and serine protease inhibitor 2.1 (Spi 2.1). A functional biological system has been developed for the PRLR that uses the promoter of the ovine β-lactoglobulin gene (5, 6). Moreover, several findings suggest that β-lactoglobulin gene is likely to be a GH-responsive gene: (*i*) *in vivo* GH has lactogenic effects in domestic dairy species, resulting in an increase in milk production (7); (*ii*) GHR mRNA has been found in the rabbit and the bovine mammary gland (8–10); and (*iii*) GHR is detected in the rat mammary gland by immunochemistry (11).

The gene encoding Spi 2.1 is a member of a group of rat genes (Spi 2.1, 2.2, and 2.3; formerly called Spi.1, Spi.2, and Spi.3 and recently redesignated *Spin2a*, *Spin2b*, and *Spin2c*) that belong to the Spi family and are expressed in the liver in a hormonally controlled manner. The transcription of Spi 2.1 gene is activated by GH in hepatocytes, but GH has a very weak effect when used alone. Glucocorticoids, which have no effect *per se*, act together with GH as a potentiating factor (12).

The putative GH-responsive region of the promoter of the genes for β-lactoglobulin and Spi 2.1 was coupled to the reporter gene encoding chloramphenicol acetyltransferase (CAT). Since CAT is a bacterial enzyme that is not found in mammalian cells, it is an ideal reporter gene to measure hormonal effects.

Two functional biological systems were developed by transiently cotransfecting a mammalian cell line in which there is no detectable level of endogenous GHR, Chinese hamster ovary cells (CHO), with the cDNA of the GHR and constructs containing either the promoter regions of the β-lactoglobulin gene or of the Spi 2.1 gene linked to the coding sequence of the CAT gene (β-lactoglobulin/CAT or Spi/CAT constructs); GH was able to activate the transcription of both promoters. These tests are used to identify regions of the GHR that are important for signal transduction.

MATERIALS AND METHODS

Transient Transfection of CHO Cells. CHO-K1 cells were grown in Ham's F-12 containing 10% (vol/vol) fetal calf serum (GIBCO) on 60-mm culture dish. Twenty-four hours before transfection, cells were washed with Ham's F-12, and

Abbreviations: GH, growth hormone; hGH, human GH; GHR, GH receptor; PRL, prolactin; PRLR, PRL receptor; Spi, serine protease inhibitor; Dex, dexamethasone; CAT, chloramphenicol acetyltransferase; IL-6, -3, and -5, interleukins 6, 3, and 5; GM-CSF, granulocyte/macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

GC3 medium was added (13). Cells were transfected at 50% confluence by the calcium phosphate precipitation procedure (14) with 3 μg of pCH110 (β-galactosidase expression vector from Pharmacia), 1.5 μg of the fusion gene construct containing the bacterial CAT coding sequence linked to either 4 kb of the β-lactoglobulin promoter (plasmid pBJ23) (5) or to the sequence from −175 to +59 of the Spi 2.1 promoter (plasmid pEMBL19) (12) and 3 μg of an expression vector [pLM108, (provided by G. Norstedt, Stockholm) (4, 15) containing the cDNA of either the wild-type GHR or a mutated GHR] (16). Note that the numbering system for these mutants is different from that previously published (16), since the numbers represent amino acid positions of the mature receptor. The total amount of DNA transfected was made up to 10 μg with carrier plasmid DNA. Cells were incubated at 37°C in 3 ml of Opti-MEM medium (GIBCO) containing the calcium phosphate-precipitated DNA. After 4 hr, the precipitate was removed, cells were subjected to 16% (vol/vol) glycerol shock for 2 min, and fresh GC3 medium containing the appropriate hormone [recombinant human GH (hGH) provided by Serono (Geneva) or dexamethasone (Dex) purchased from Sigma, or both] was added. The cells were collected by scraping 72 hr after transfection. Cell extracts were prepared by three consecutive freeze/thaw cycles followed by centrifugation at 15,000 × g for 10 min. The supernatant was used for CAT assay, and the pellet was resuspended in 25 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$. Aliquots of the supernatants were normalized for β-galactosidase activity (17).

Transient Transfection of COS Cells. COS-7 cells were grown in MEM containing 10% fetal calf serum on 30-mm culture dish. Cells were transfected at 70% confluence by a modification of the DEAE-dextran–chloroquine procedure (18) with 1 μg of pLM108 containing the cDNA of either wild-type GHR or mutated GHR: the precipitate was made with 10 mg of DEAE-dextran per ml, 100 μM chloroquine, and 10 ng of DNA per μl. After 4 hr, the precipitate was removed, cells were subjected to 10% dimethyl sulfoxide shock for 2 min, and fresh medium was added. Forty-eight hours after transfection, the medium was removed and cell surface GH binding was studied.

Mutant Constructions. cDNAs encoding truncated (T) mutants ending at positions 276 and 436 (T276 and T436) were generated as described (16). cDNA encoding the deletion mutant "Δ279-293" was created by using a 20-mer oligonucleotide (5′-GAAGATCTGGCATCTTAATC-3′) and the PCR to splice out the region. cDNAs encoding the substitution mutants were created by using PCR to replace the wild-type sequence: an upstream 20-mer covering the *Eco*RV restriction site at codon 153 and downstream 40- to 58-mers covering the *Bgl* II restriction site at codon 301 and the codons mutated to give proline-to-alanine changes, yielding mutants called P282A (CCC → GCC/G); P283A, P285A, and P287A (CCA → GCA/G); and 4P/A, which included the four previous mutations. The PCR fragments containing the mutations were sequenced in both directions to verify the presence of the mutations (19).

Hormone Binding. ^{125}I-labeled hGH (^{125}I-hGH) was prepared by using chloramine T with a specific activity ranging from 80 to 140 μCi (1 μCi = 37 GBq)/μg. ^{125}I-hGH (50–100 pM) was incubated with 300 μg of membrane protein from control or transfected CHO-K1 cells in 300 μl of 25 mM Tris·HCl, pH 7.4/20 mM $MgCl_2$/0.1% bovine serum albumin (BSA). Incubations were carried out at 4°C for 20 hr. Bound and free hormones were separated by filtration (GVWP Millipore filters). To measure cell surface binding, 50–100 pM ^{125}I-hGH was incubated with 4 × 10^5 cultured COS-7 cells in phosphate-buffered saline (PBS) containing 0.5% BSA. Incubations were carried out at 8°C for 20 hr. Cells were washed twice with ice-cold PBS; then 1 ml of 1 M NaOH was added, and cell suspension was counted in a γ counter.

CAT Assay. CAT assays were performed for 90 min as described (20) with 0.1 μCi of [^{14}C]chloramphenicol (Amersham). Fold induction of CAT activity (in the presence of hormone) was calculated from basal level (in the absence of hormone).

Statistical Analyses. Statistical analyses were performed by using Duncan's multiple range test.

RESULTS

Functional Expression of Rat GHR in Transiently Transfected CHO Cells. CHO cells were transiently cotransfected with plasmids containing the GHR and either β-lactoglobulin/CAT or Spi/CAT. Concentrations of the plasmids required for optimal activation were determined. The best results were obtained with a ratio of 1:2 of promoter/CAT to receptor plasmid. In the absence of receptor plasmid, hGH did not induce CAT activity; the fold induction was 1.0 ± 0.1 with β-lactoglobulin/CAT and 1.3 ± 0.2 with Spi/CAT (see Fig. 4). The use of serum-free conditions in the cotransfection experiments resulted in a relatively low basal CAT activity when the cells were cultured in the absence of GH (2% CAT conversion with β-lactoglobulin/CAT and 2.4% with Spi/CAT).

Functional Activity of Rat GHR on Milk Protein Gene Transcription. We have used the construct β-lactoglobulin/CAT to test the ability of GH to activate β-lactoglobulin gene transcription. CHO cells grown in serum-free medium were transiently cotransfected with the β-lactoglobulin/CAT construct and an expression vector containing the rat GHR cDNA. This cotransfection results in a clearly inducible CAT activity by GH, with a dose-dependent response (Fig. 1) and an $ED_{50} \approx 0.35$ nM hGH. CAT activity began to increase at

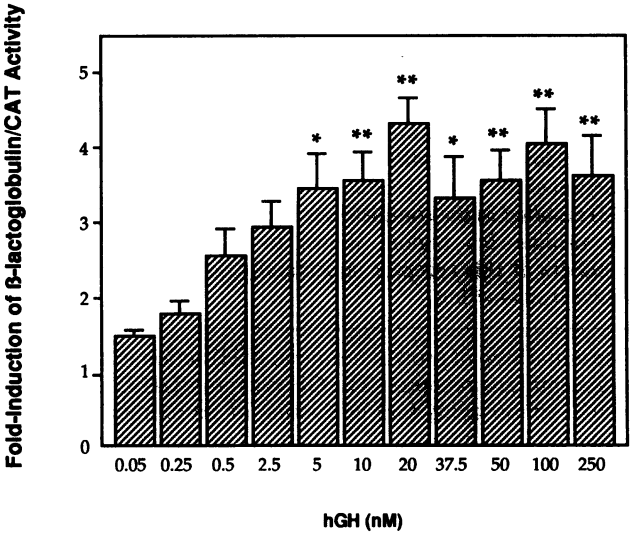

FIG. 1. Effects of increasing concentrations of hGH on the expression of β-lactoglobulin/CAT in CHO cells. CHO cells were transiently cotransfected with a plasmid containing the GHR and the fusion construct β-lactoglobulin/CAT. Cells were pretreated as described in text, and CAT activity was measured at various hGH concentrations. The fold induction was calculated as the percent chloramphenicol conversion in the presence of GH divided by the percent chloramphenicol conversion in the absence of GH. Results represent the mean ± SEM of 4–10 independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with values at 0.05 nM hGH. In addition, the value at 0.25 nM is significantly different from that at 10, 50, and 250 nM ($P < 0.05$) and at 20 and 100 nM ($P < 0.01$), while the value at 0.5 nM is significantly different from that at 20 and 100 nM ($P < 0.05$).

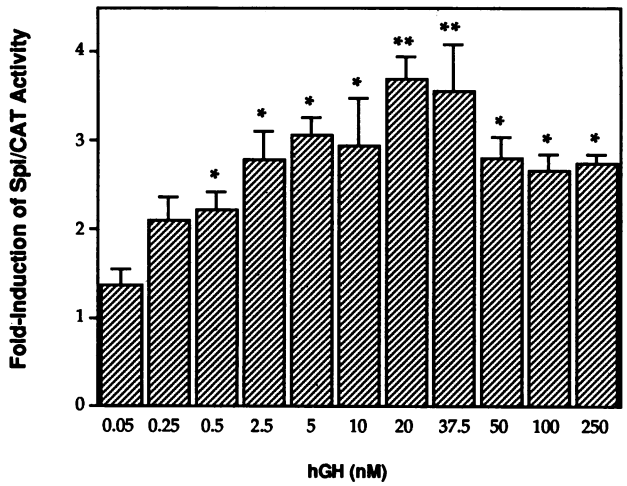

FIG. 2. Effects of increasing concentrations of hGH on expression of Spi/CAT in CHO cells. CHO cells were transiently cotransfected with a plasmid containing the GHR and the fusion gene Spi/CAT. Cells were pretreated as described in the legend to Fig. 1, except that cells were cultured in the presence of 250 nM Dex. Results are means ± SEM of 3–14 independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with values at 0.05 nM hGH. The value at 0.25 nM is significantly different from that at 20 and 37.5 nM ($P < 0.05$), and the value at 20 nM is significantly different from that at 100 nM ($P < 0.05$).

0.5 nM hGH and became significant at 5 nM hGH with a maximal effect at 20 nM hGH (4.3 ± 0.3-fold induction), after which the response was relatively stable. No decline was observed in CAT activity even at high hGH concentrations.

Dex (250 nM) had no effect alone on the expression of β-lactoglobulin/CAT. In addition, when combined with GH, Dex did not potentiate GH action (data not shown).

Functional Activity of Rat GHR on Spi 2.1 Gene Transcription. CHO cells grown in serum-free medium were transiently cotransfected with the Spi 2.1 (−175/+59)/CAT construct and with an expression vector containing the rat GHR cDNA. Neither GH nor Dex had *per se* significant effects on CAT activity, but Dex and GH together stimulated CAT activity. The effects of increasing hGH concentrations on CAT activity were tested in the presence of 250 nM Dex, and a dose-dependent response was obtained (Fig. 2) with an $ED_{50} \approx 0.15$ nM hGH. The effect became significant at 0.5 nM hGH, with a maximal effect at 20 nM hGH. The fold induction was 3.7 ± 0.3 compared with the control without hormone, and 2.8 ± 0.3 when compared with cultures containing Dex alone. In contrast to β-lactoglobulin/CAT, there was a significant decrease (30%) in the response at 100 nM. The effect of increasing concentrations of Dex on Spi/CAT transcription was tested in the presence of an optimal concentration of hGH (20 nM). Dex, alone, had no significant stimulatory effect, and in the presence of hGH the optimal Dex concentration was 250 nM. This concentration was used in subsequent experiments.

Functional Activity of Mutants of the GHR. Two truncated cDNAs (T276 and T436), a deletion mutant of Box I (Δ279-293), and mutants with proline-to-alanine changes in Box I (P282A, P283A, P285A, P287A, and 4P/A) of the rat GHR (Fig. 3) were introduced in the pLM108 expression vector

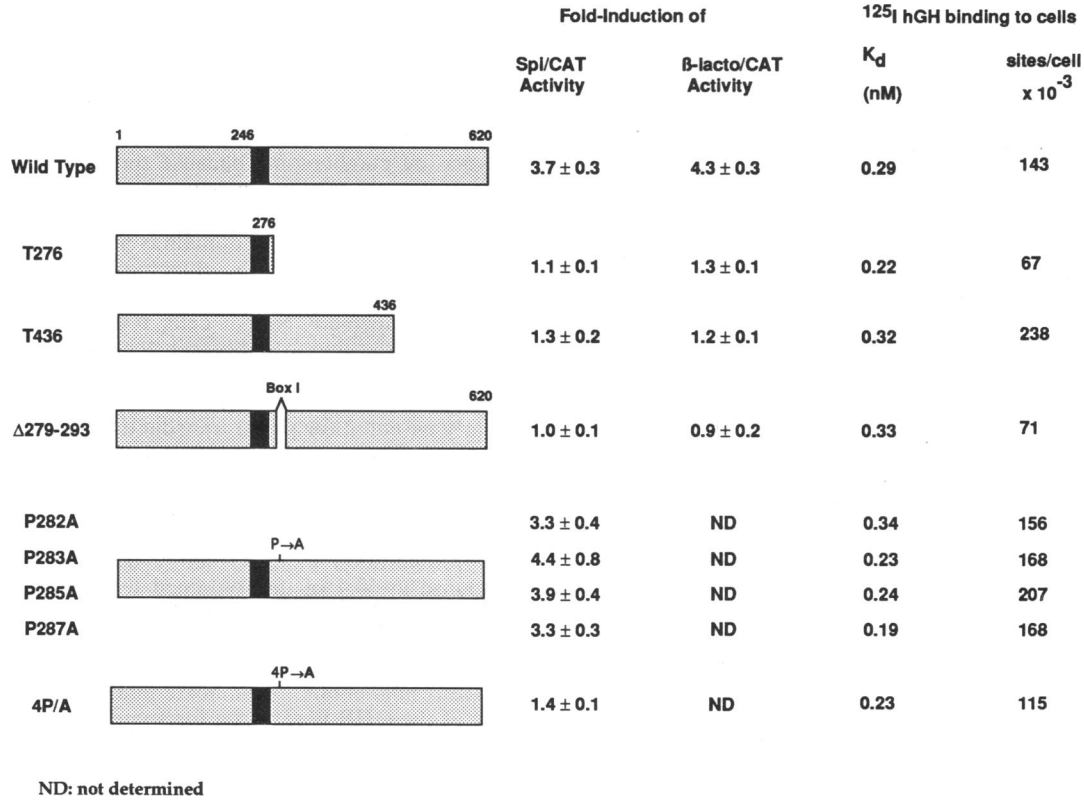

FIG. 3. Schema of wild-type and mutant forms of the GHR tested in the functional assays. The wild-type receptor consists of 620 aa with a single transmembrane domain region (black). Truncated mutants (T276 and T436) have 6 and 166 aa in their cytoplasmic domain, respectively. The mutant Δ279-293 has 15 aa deleted in the cytoplasmic domain. The substitution mutants have one proline mutated to alanine in Box I; the 4P mutant has all four prolines (282, 283, 285, and 287) replaced by alanine. The functional activity (fold induction of CAT activity) of each mutant is shown. Results represent the mean ± SEM of 5–14 independent experiments. All mutants were tested in the Spi/CAT functional test. The mutated forms T276, T436, and Δ279-293 were tested in the β-lactoglobulin/CAT test. Characteristics of cell surface binding of ^{125}I-hGH to wild-type and mutant forms are shown.

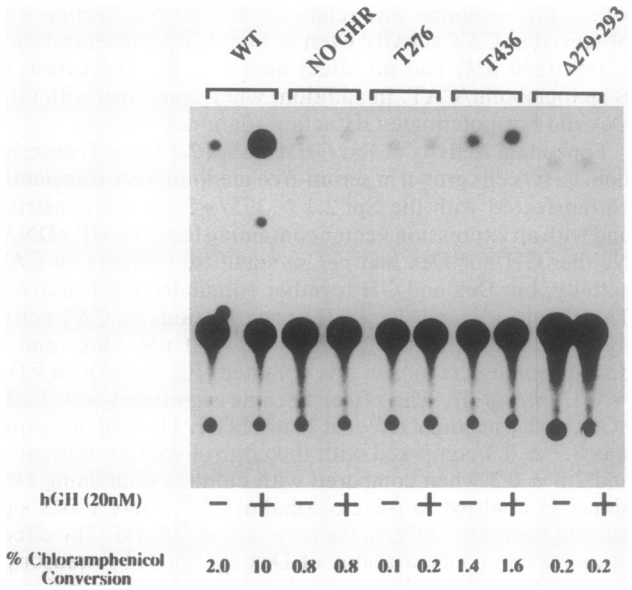

FIG. 4. Comparison of biological activity of mutant forms of the GHR in the β-lactoglobulin/CAT assay. CHO cells were transiently cotransfected with plasmids containing cDNAs encoding either the wild-type GHR (lanes WT) or mutated form of GHR (lanes T276, T436, or Δ279-293) and the fusion construct β-lactoglobulin/CAT. Lane "NO GHR" contained CHO cells transfected with the fusion construct β-lactoglobulin/CAT alone. hGH (20 nM) was added to (+) or omitted from (−) the serum-free medium during the experiment. Acetylated products of chloramphenicol are displayed after TLC and autoradiography.

and transfected into CHO cells with either β-lactoglobulin/CAT (Fig. 4) or Spi/CAT constructs. The truncation mutants (T276, T436), the deletion mutant (Δ279-293), and the quadruple alanine substitution mutant (4P/A) were unable to transmit a signal and activate the promoter/CAT constructs, whereas individual alanine substitution mutants (P282A, P283A, P285A, and P287A) retained full activity.

Specific binding of ^{125}I-hGH to cultured COS cells that were transiently transfected with either the wild-type GHR or the mutated forms demonstrated that these mutant receptors were normally expressed on the surface of the transfected cells. In Fig. 3 are presented the dissociation constants and the maximum binding capacities obtained for the different forms of the GHR by Scatchard plot analyses of competition experiments. In addition, specific binding of ^{125}I-hGH to membranes of transiently transfected CHO cells with either the wild-type GHR or the mutated forms ranged from 2.8% to 3.1% of the radioactivity (per 300 μg of membrane protein). These results show that the mutant receptors were appropriately expressed in CHO cell membranes to a level similar to that of wild-type GHR.

	279 293
GHR	LILPPVPVPKIKGIDP
PRLR	CIFPPVPGPKIKGFDA
gp130	HIWPNVPDPSKSHIAQ
GMCSFR-β	KMEEKIPNPSKSHFLQ
GCSFR	PLWPSVPDPAHSSLGS

FIG. 5. Sequence alignment of a part of the cytoplasmic domain of the GHR with other GH/PRL/cytokine receptor family members. The GHR is numbered from the first residue of the mature protein. The four prolines of GHR Box I are underlined. The proline-rich regions of the following molecules are aligned: PRLR; gp130, β subunit of the IL-6 receptor; GMCSFR-β, common β subunit of GM-CSF, IL-3, and IL-5 receptors; and GCSFR, G-CSF receptor.

DISCUSSION

We have used the promoters of genes encoding β-lactoglobulin and Spi 2.1 to establish bioassays based on specific gene activation by GH. These tests are relatively rapid, as they involve transient transfections of cells. Our findings demonstrate that the β-lactoglobulin gene is inducible by GH in CHO cells transfected with GHR cDNA, indicating that a promoter for a milk protein gene is sensitive to GH, even when it is introduced into nonmammary cells. A second functional test uses the region −175/+59 of the promoter of the Spi 2.1 gene. A GH cis-acting response element was characterized in the promoter region of this gene. Paquereau *et al.* (21) transfected constructs containing different parts of the Spi 2.1 promoter fused to the coding sequence of the CAT gene in hepatocytes. The −175/+59 fragment showed the highest fold induction of CAT activity in the presence of both Dex and GH. The same sequence of the promoter was used in our assay. Our results demonstrate that the Spi 2.1 gene is inducible by GH in CHO cells transfected with the GHR cDNA, indicating that CHO cells contain some of the appropriate downstream elements, including nuclear transcription factors (22) rendering these cells suitable for such a bioassay.

The optimal hGH concentration is the same in both tests, and the ≈4-fold induction obtained is similar. These tests are thus suitable to study structure–function relationships of GHR.

Billestrup *et al.* (3) described a system that takes advantage of GH-mediated effects on insulin-secreting cells. In this system, RIN-5AH cells, stably transfected with the cDNA encoding the GHR, were shown to have an increased GHR expression accompanied by an increased responsiveness to GH, resulting in a stimulation of insulin production. Emtner *et al.* (4) demonstrated GH-stimulated protein synthesis in CHO cells stably expressing GHR. Möller *et al.* (23) recently showed that CHO cells stably transfected by the GHR cDNA responded to GH by changes in cell proliferation and activation of MAP kinase. However, all of these functional assays are cumbersome because they are based on stable transfection of eukaryotic cells with GHR cDNA.

The truncated mutant T276 that contains only five of the 350 aa in the intracellular domain is not able to stimulate the transcription of either the β-lactoglobulin or the Spi 2.1 construct. This mutant was also inactive in the other tests: in stably transfected RIN-5AH cells, it did not stimulate insulin production (3); and in stably transfected CHO cells, it failed to stimulate mitogenesis or activate mitogen-activated protein kinase (23). Thus, the intracellular domain of GHR is clearly essential for signal transduction.

Mutant T436, with 184 aa deleted in the intracellular domain, is also not active in the promoter tests, and it did not stimulate insulin production in RIN-5AH cells (4). However, it was able to activate mitogen-activated protein kinase and cell proliferation in CHO stably transfected, with a GH response to 60% of that obtained with the wild-type GHR (23). There are some potential explanations for such a divergence: intermediate effects would not be easy to detect in our tests because of the amplitude of the response (≈4-fold). These results could also indicate that the carboxyl-terminal part of the GHR is required for some but not all functions. Finally, the cytoplasmic domain could contain more than one sequence for signal transduction, with different regions being involved in different GH effects.

Expression of the mutant Δ279-293, with the Box I deleted, resulted in the complete absence of GH activation in both tests. The 15 aa deleted contain the proline-rich region. Replacement of the four proline residues of Box I by alanine demonstrated their importance for signal transduction. Interestingly, individual proline-to-alanine mutations did not affect the activity of the GHR. More detailed mutagenesis

will enable us to determine which specific proline residues are involved. This proline-rich region is totally conserved between rat, man, rabbit, sheep, cow, and mouse GHR, and only 1 aa differs in the chicken GHR. This region has an 80% identity with the PRLR with complete conservation of the four proline residues (Fig. 5). A mutant of PRLR with Box I deleted has been shown to be unable to activate the β-lactoglobulin gene transcription (unpublished data). The GHR shows limited similarity of Box I with other members of the cytokine receptor family (Fig. 5). Box I has been shown to be essential for the functional activity of the interleukin (IL) 6 (IL-6) signal-transducing glycoprotein gp130 (24), the granulocyte colony-stimulating factor (G-CSF) receptor (25), and the common β subunit of receptors for granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-3, and IL-5 (26). Within Box I, a Pro-Xaa-Pro motif (amino acid residues 285–287 of the GHR) is present in all members of the family (except the IL-7 receptor). Replacement of these two proline residues by serine in gp130 resulted in the loss of functional activity, demonstrating the importance of this motif for another member of this family (24). This sequence may be important in the tertiary structure of the protein because proline is considered to be a helix breaker. Box I could be associated with a common or similar downstream molecule, such as the tyrosine kinase, JAK2, which could be the activator of signal transduction [Box I presents some similarity with the Src homology 3 (SH3) binding sites of SH3-binding proteins. SH3 binding site consists of a sequence of 10 aa very rich in proline residues (Thr-Met-Pro-Pro-Pro-Leu-Pro-Pro-Val-Pro) (27)]. SH3 domains have been identified as conserved sequences within the Src family of tyrosine kinases. They are found in many proteins involved in intracellular signal transduction and are part of specific protein–protein interactions (28).

The results presented in this paper illustrate that the approach using cotransfection of the receptor cDNA and a target reporter gene is valid for the GHR. The mechanisms by which the GH signals are transferred inside the cells remain unclear, since none of the classical hormonal second messengers appears to be involved in this process. More detailed mutagenesis of the GHR will enable us to continue to determine precise functional domains of the GHR using the functional tests that are presented here. Since GH has multiple direct and indirect actions and probably several signalling pathways, the development of other functional tests will be important to better understand the process of GH signal transduction.

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