Insulin-like growth factor I (IGF-I), a growth hormone (GH)-dependent growth factor, exerts feedback regulation of GH by inhibiting GH gene expression. IGF-I inhibition of GH secretion is enhanced 3-5-fold in GC rat pituitary cells overexpressing the wild type 950Tyr human IGF-I receptor which autophosphorylates appropriately. To determine the critical amino acid residue responsible for IGF-I signaling, insertion, deletion, and site-directed mutants were constructed to substitute for 950Tyr in exon 16 of the human IGF-I receptor β-subunit transmembrane domain. All mutant transfectants bound IGF-I with a similar Kd to untransfected cells but had markedly increased (7-34-fold) IGF-I-binding sites. GH responsiveness to IGF-I was tested in mutant transfectants. Overexpressed site-directed and insertion mutant IGF-I receptors exhibited a modest suppressive effect on GH in response to the IGF-I ligand, similar to that observed in untransfected cells. Deletion mutant (IGFIR Δ22) (amino acid 944-965) did not transduce the IGF-I signal to the GH gene. Site-directed and insertion mutants therefore did not enhance the IGF-I response of the endogenous rat receptor, unlike the 950Tyr wild type transfectants which enhanced the IGF-I signal. All mutant transfectants, except the deletion mutant, internalized radioactive ligand similarly to 950Tyr wild type transfectants. 950Tyr of the human IGF-I receptor is therefore required for IGF-I signal transduction in the pituitary somatotroph, but not for IGF-I-mediated internalization.

Experimental Procedures

Materials-Recombinant human IGF-I (Met-59) was kindly provided by Fujisawa Pharmaceutical Co (Osaka, Japan). IGF-I was purchased from Amersham Corp. α-IR3 was purchased from Oncogene Sciences (Manhasset, NY).

Cell Culture—GC rat pituitary cells secreting rat GH were grown at 37 °C in a humidified atmosphere of 95% air, 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum.

Mutagenesis of the Human IGF-I Receptor cDNA—The wild type human IGF-I receptor expression plasmid (pRSV-IGFIR) has been previously described (6). A Smal-HindIII fragment from pRSV-IGFIR was subcloned into M13 mp19. The following primers were utilized for mutagenesis, 5'-AACCCGAGTTCCTTCAGGCTC-3' (Cys), 5'-AACCCGAGTTCCTTCAGGCTC-3' (Ser), 5'-AACCCGAGTTCCTTCAGGCTC-3' (Ala), 5'-AACCCGAGTTCCTTCAGGCTC-3' (Leu), 5'-AACCCGAGTTCCTTCAGGCTC-3' (Thr), and 5'-GGGAATGGAGTGTTTCAGCGCT-3' (22 amino acid deletion). Mutant oligonucleotides were annealed to the single-stranded DNA, and subsequent mutagenesis was performed utilizing an Amersham kit according to the manufacturer's instructions. After reintroduction of the mutant sequence into the pRSV-IGFIR vector, all constructs were subjected to dideoxy sequencing utilizing T7 sequence (U. S. Biochemical) to confirm the mutant sequences. A Sall linker (5'-GTCGAC-3') was inserted into the SacI site of Smal-HindIII fragment of the IGF-I receptor to produce IGF-I CRH containing three additional amino acids (Cys-Arg-His) at position 950. All plasmids were prepared on two CsCl gradients.

Stable Transfection into GC Rat Pituitary Cells—Semiconfluent GC cells in 100-mm dishes were cotransfected by the CaPO4 method (12) using a 10:1 ratio of linearized mutant IGF-I receptor plasmid to pSV2neo. 16 h after transfection, the cells were shocked with 15% glycerol for 2 min, washed, and incubated in serum-containing media.
dium for 24 h. Cells were then split 1:4, and neomycin (G418) was added in fresh medium at a concentration of 400 μg/mL. Medium was replenished every 72 h. G418-resistant colonies were subcloned and further characterized.

**Southern Blot**—Southern hybridization was performed as previously described (6). Briefly, genomic DNA of the mutant transfectants was extracted and digested with EcoRI. The DNA fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to a nylon membrane. Membrane was baked, prehybridized, and hybridized with a 32P-labeled IGF-I receptor cDNA (specific activity 4 x 10^6 cpm/μg). The membrane was washed and autoradiographed for 24 h at -70°C using Fuji film and intensifying screens.

**IGF-I Binding**—Binding of radiolabeled IGF-I was performed in suspension. Cells (10^6) were incubated with ^125^I-IGF-I (50,000 cpm, specific activity, 2,000 Ci/mmol) and increasing concentrations of unlabeled IGF-I or α-IR3 (anti-human IGF-I receptor antibody) in a final volume of 1 ml of binding buffer (50 mM HEPES buffer (pH 8.0), 1% bovine serum albumin, 150 mM NaCl, 1.2 mM MgSO_4_) at 15°C. Nonspecific binding was defined as the binding observed in the presence of excess (100 nM) unlabeled IGF-I. At the end of the 3-h incubation period, cells were centrifuged, and cell-associated radioactivity of the samples was determined as described in IGF-I binding.

Degraded ^125^I-IGF-I was assessed by trichloroacetic acid precipitation (TCA) and autoradiography. Cell-associated radioactivity was separated from free ^125^I-IGF-I by adding 300 μl of ice-cold dibuthylphthalate. Cell-associated radioactivity of the samples was then determined by gamma-counting. Calculation of total bound ligand included the free-labeled ligand.

**GH Secretion**—5 x 10^5_ cells were plated on 9-cm² multiwells and grown for 24 h in the growth medium. Medium was then aspirated and replenished with 1.5 ml of serum-free defined medium (13) with or without 6.5 nM IGF-I. Aliquots of medium were removed at the indicated time points and assayed for rat GH using radioimmunoassay reagents supplied by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD).

**IGF-I Internalization**—Cells (10^6) were incubated with ^125^I-IGF-I (40,000 cpm) in a final volume of 0.5 ml of Dulbecco's modified Eagle's medium (pH 7.8) with 1% bovine serum albumin and 10 mM HEPES. At the end of the 30-min incubation period at 37°C, the suspension was acidified by the addition of 1 N HCl (30 μl) (14). After 6 min on ice, cells were centrifuged at 4°C, intracellular (acid-resistant) or cell-associated ^125^I-IGF-I was determined as described in IGF-I binding. Degraded ^125^I-IGF-I was assessed by trichloroacetic acid precipitation (10%) of 300 μl of incubation buffer. After 4 min at 4°C, the degraded ^125^I-IGF-I in the supernatant was determined by gamma-counting (15).

% Internalization = intracellular + degraded

after subtraction of respective values for untransfected control cells (16).

**Tyrosine Phosphorylation**—Cells labeled for 16 h with [35S]methionine were washed with phosphate-buffered saline and incubated with or without IGF-I (6.5 nM) in serum-free defined medium for 1 min at 37°C. Cells were then lysed with lysis buffer (1% Triton X-100, 30 mM sodium pyrophosphate, 10 mM Tris (pH 7.6), 5 mM EDTA (pH 8), 50 mM NaCl, 0.1% bovine serum albumin, 2 mM sodium orthovanadate, 200 mM phenylmethylsulfonyl fluoride) and centrifuged for 15 min at 3000 × g. Immunoprecipitation was performed using a monoclonal anti-phosphotyrosine antibody (Ab-2, Oncogene Science, Manhasset NY) at 4°C for 3 h. Protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) and anti-mouse IgG (Sigma) were incubated with the monoclonal anti-phosphotyrosine antibody cell lysate complex for 2 h at room temperature. Immunoprecipitates were washed six times in lysis buffer, resuspended in sample buffer, boiled for 5 min, and electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gels.

**RESULTS AND DISCUSSION**

Mutant human IGF-I receptors are depicted in Fig. 1. For these experiments, ^90^Tyr was replaced with either Cys, Ser, Ala, Leu, or Thr (site-directed mutations). IGFR-CRH consisted of three additional amino acids (Cys-Arg-His) inserted at position 950. 22 amino acids (from 944 to 965) were deleted in IGFR Δ22. GC rat pituitary cells were stably transfected with these mutant IGF-I receptor cDNAs (6). After 1 month of neomycin selection, binding of ^125^I-IGF-I was assessed in untransfected cells and in all mutant stable transfectants. Only clones exhibiting at least 7-fold increase in maximal specific binding were selected for further study. Fig. 2A shows that increasing amounts of unlabeled IGF-I displaced cell-associated ^125^I-IGF-I binding to all cells, with 50% displacement of maximum binding achieved by 0.6–2 nM IGF-I. When these binding displacement data were subjected to Scatchard analysis, a linear plot was obtained for all mutant transfectants, indicating the presence of a single class of high affinity receptors in these pituitary cells (Fig. 2B). The derived association constant (K_d) for ^125^I-IGF-I binding was similar in all transfected cells, ranging from 0.25 to 0.66 nM. This similarity of ligand affinity for the different mutant receptors is not surprising as the configuration of the extracellular domain (α-subunit) is expected to remain intact when the β-subunit was mutated. The derived number of mutant IGF-I receptors present on each transfectant was increased from 7- to 34-fold compared to untransfected cells, as shown in Fig. 2C.

To confirm integration of exogenous human IGF-I receptor into rat GC cell genomic DNA, Southern blot analysis of transfected cell DNA was performed. The results of DNA hybridizations obtained from the mutant-transfected and untransfected cells are shown in Fig. 3. The human receptor probe hybridized appropriately to all transfected DNA samples but only hybridized minimally to DNA extracted from untransformed GC cells, confirming that the exogenous human IGF-I receptor cDNAs were integrated into the genomic DNA of these cells. The major 5-kilobase DNA fragment that was expected to be released from pRSV-IGFIR by digestion with EcoRI was visualized in each transfectant, in addition to randomly sized DNA bands.

Synthesis of the mature α2β2 tetrameric IGF-I receptor is initiated by dimerization of the αβ proreceptor precursors (7, 17). Overexpressing the human IGF-I receptor in rat cells, therefore, may facilitate the formation of hybrids between the endogenous rat αβ receptor and the exogenous human αβ half-receptor (18, 19). In an attempt to quantitate endogenous rat IGF-I receptors present in the transfectants, we performed ^125^I-IGF-I binding in the presence of increasing amounts of the monoclonal antibody for the human IGF-I receptor, α-
**Fig. 2. Summary of $^{125}$I-IGF-I binding to mutant transfectants.** Cells were incubated with 50,000 cpm $^{125}$I-IGF-I together with increasing amounts of unlabeled IGF-I. Binding was performed in suspension at 15 °C for 3 h. Panel A, $^{125}$I-IGF-I displacement by unlabeled IGF-I. Values were standardized to maximum binding which ranged from 5% in untransfected cells to 55% in mutant transfectants. Each point represents the mean of three determinations, representative of two or three separate experiments. Panel B, Scatchard analysis of IGF-I binding. Data obtained from displacement curve were subjected to Scatchard analysis. The derived slope for untransfected cells is shown in the inset. Calculated bound IGF-I includes $^{125}$I-IGF-I. B/F, bound to free ratio. Panel C, The $K_d$ values and binding sites/cell were derived from the Scatchard analysis.
IR3. α-IR3 has been shown to specifically inhibit $^{125}$I-IGF-I binding to human IGF-I receptors, but not rat IGF-I receptors (20). Using excess concentrations of α-IR3 (>1 μM), a residual 7% nondisplaceable $^{125}$I-IGF-I binding was still present on transfected cells (Fig. 4). This suggests that despite overexpression of the transfected human IGF-I receptor, a small population of rat/human hybrid receptors and/or endogenous rat/rat holotetrameric receptors are also present. Although α-IR3 failed to compete for labeled IGF-I binding to endogenous receptors in untransfected heterologous Chinese hamster ovary cells (21), we cannot definitively conclude that residual nondisplaceable binding in $^{95}$Tyr wild type (WT) GC transfectants represents human/rat hybrid receptors. We have recently demonstrated that most of the endogenous rat IGF-I receptors present on transfected GC cells formed hybrids with the overexpressed human IGF-I receptors. Therefore, the residual nondisplaceable binding may represent a predominant population of human/rat hybrid receptors. Although the α-IR3 displacement experiment was performed using $^{95}$Tyr (WT) transfectants, similar hybrid formation presumably occurred in the other mutant receptor transfectants.

To analyze the capability of the mutant IGF-I receptors to transduce the IGF-I signal to the GH gene, cells were treated with IGF-I (6.5 nM) and GH secretion was measured. Previous dose-response experiments had demonstrated that 6.5 nM IGF-I maximally attenuates somatotroph GH secretion with no further GH suppression observed by increasing concentrations of IGF-I (6). Furthermore binding studies showed that 6.5 nM IGF-I occupies more than 90% of the available binding sites on these mutant cells. Incubation of the cells overexpressing $^{95}$Tyr (WT) receptor with IGF-I (6.5 nM) resulted in a 61% suppression of GH observed after 24 h of IGF-I treatment compared to a modest suppression of GH observed in untransfected cells ($p < 0.001$) (Fig. 5). Mutant transfectants responded to IGF-I similarly to untransfected cells, with GH secretion suppressed only by 20%. Δ22 transfectants, however, exhibited no suppression of GH, despite the presence of the endogenous rat or hybrid IGF-I receptors. Therefore although overexpression of intact WT IGF-I receptor in GC cells enhanced IGF-I signaling, all mutant receptor transfectants failed to augment the IGF-I signal. $^{95}$Tyr, therefore, is required for IGF-I signal transduction to the GH gene.

Most endogenous rat IGF-I receptors in these cells may have formed hybrids with the overexpressed transfected human IGF-I receptors. The deletion mutant (IGFIR Δ22) failed to inhibit GH secretion in response to IGF-I despite the presumed presence of intact rat/Δ22 human hybrids. Interestingly, truncated platelet-derived growth factor receptors have been demonstrated to inhibit receptor autophosphorylation when forming a dimer with intact platelet-derived growth factor receptor (22). Conclusions regarding hybrid receptor function from the data shown should be interpreted with caution, as the IGFIR Δ22 construct results in a rela-

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IGF-I Suppresses Pituitary GH Secretion

**Fig. 5.** Time course of IGF-I action on GH secretion in untransfected cells and transfectants. Cells were treated with 6.5 nM IGF-I for the indicated periods. GH (% of control) represents relative amounts of GH secreted by treated cells compared to hormone secreted by the same cells not treated with IGF-I. Each value represents the mean of two or three separate experiments each with six determinations. All values for 950Tyr (WT) transfectants were lower than those from mutant receptor transfectants and untransfected cells (p < 0.001). ○, untransfected; □, IGFIR-950Tyr (WT); ◇, IGFIR-900Cys; +, IGFIR-950Ala; ●, IGFIR-950Ser; ▲, IGFIR-950Thr; ●, IGFIR-CRH; △, IGFIR-Δ22.

Effectively gross mutation, compared with the site-specific receptor mutations.

Exon 16 of the insulin receptor interacts with IRS-1, a putative cellular messenger for transduction of the insulin signal (10). Furthermore, phosphorylation of IRS-1 was also reduced by mutations of 960Tyr and 955Ser in the insulin receptor (8–10). Interestingly, this mutant insulin receptor underwent β-subunit autophosphorylation in response to insulin binding, indicating that receptor autophosphorylation is not in and of itself sufficient for phosphorylation of cytoplasmic protein (8, 9). Therefore, the transmembrane domain around 950Tyr in the insulin receptor appears to be important for interaction with the IRS-1 molecule to transduce the insulin signal. IGF-I has also been shown to induce phosphorylation of IRS-1 in intact cells (23–25), and exon 16 of the IGF-I receptor may therefore interact with IRS-1 to facilitate IGF-I signaling.

To evaluate whether or not the transfected receptor nevertheless retained its ability to autophosphorylate as well as to phosphorylate endogenous pp183 (10), cells were treated with IGF-I for 1 min after metabolic labeling. Fig. 6 shows that 950Tyr does in fact contain phosphorylated tyrosine residues (97 kDa) which are immunoprecipitated by monoclonal anti-phosphotyrosine antibody, as well as a larger phosphorylated protein corresponding to the predicted size of pp183. As expected, the 135-kDa α-subunit is also immunoprecipitated, indicating the integrity of the covalent bonding of the receptor subunits prior to resolution. The site-directed mutant, 950Ala, also demonstrated a similar phosphorylation pattern, albeit of lesser intensity.

950Tyr of the IGF-I receptor β-subunit is situated within an asparagine-proline-X-tyrosine (NPXY) motif. This sequence has been determined to be required for low density lipoprotein receptor internalization (11). We therefore tested the ability of IGF-I receptor mutants to internalize 125I-IGF-I. Site-directed and insertion mutants as well as the 950Tyr (WT) receptors internalized 46–65% of exogenous 125I-IGF-I, while IGFIR Δ22 failed to internalize labeled IGF-I ligand (Fig. 7). This suggests that 950Tyr is not required for IGF-I receptor internalization. Recently, the NPXY motif present in the insulin receptor has been shown not to contribute to ligand-induced receptor internalization (26), while the GXLY motif present upstream from the NPXY motif is critical for insulin receptor internalization (27). Although the GXLY motif is intact in the IGFIR Δ22, the deleted region (944–965) in IGFIR Δ22 may contain additional critical amino acid sequences for IGF-I internalization other than this motif.

In this study, we demonstrate that 950Tyr of the human insulin receptor is required for IGF-I action on GH secretion and that this mutation results in a receptor with reduced ability to autophosphorylate and to undergo tyrosine phosphorylation of IRS-1. These findings suggest that the transmembrane domain around 950Tyr of the IGF-I receptor is important for interaction with the IRS-1 molecule to transduce the IGF-I signal.
IGF-I receptor β-subunit transmembrane domain is required for IGF-I signaling to the GH gene. The inhibitory effects of IGF-I on GH gene expression require a relatively long lag-period, unlike the early metabolic effects of IGF-I, such as thymidine incorporation, glycogen synthesis, and glucose uptake which manifest within minutes (1). Although no pituitary cells lacking IGF-I receptors have been described, the cells employed for these studies provide a unique model to study IGF-I ligand-mediated signaling to the nucleus, independent of the metabolic actions of IGF-I. This model of polypeptide hormone secretion allows dynamic testing of the IGF-I signal in a physiologically relevant cell type.

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