Growth Hormone Promoted Tyrosyl Phosphorylation of Growth Hormone Receptors in Murine 3T3-F442A Fibroblasts and Adipocytes†

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ABSTRACT: Because many growth factor receptors are ligand-activated tyrosine protein kinases, the possibility that growth hormone (GH), a hormone implicated in human growth, promotes tyrosyl phosphorylation of its receptor was investigated. ^{125}I-Labeled human GH was covalently cross-linked to receptors in intact 3T3-F442A fibroblasts, a cell line which differentiates into adipocytes in response to GH. The cross-linked cells were solubilized and passed over a column of phosphotyrosyl binding antibody immobilized on protein A–Sepharose. Immunoadsorbed proteins were eluted with a hapten (*p*-nitrophenyl phosphate) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. The eluate from the antibody column contained an M_r 134 000 ^{125}I-GH–receptor complex. A similar result was obtained when the adipocyte form of 3T3-F442A cells was used in place of the fibroblast form. *O*-Phosphotyrosine prevented ^{125}I-GH–receptor complexes from binding to the antibody column, whereas *O*-phosphoserine and *O*-phosphothreonine did not. In studies of GH-promoted phosphorylation in 3T3-F442A fibroblasts labeled metabolically with [^{32}P]P_i, GH was shown to stimulate formation of a ^{32}P-labeled protein which bound to immobilized phosphotyrosyl binding antibodies. The molecular weight of 114 000 obtained for this protein is similar to that expected for non-cross-linked GH receptor. The M_r 114 000 phosphorylated protein could be immunoprecipitated with anti-GH antibody, indicating that GH remained noncovalently bound to this protein during absorption to and elution from the immobilized phosphotyrosyl binding antibody. Phosphoamino acid analysis after both limited acid hydrolysis and extensive base hydrolysis of the M_r 114 000 phosphoprotein confirmed the presence of phosphotyrosyl residues. These observations provide strong evidence that binding of GH to its receptor stimulates phosphorylation of tyrosyl residues in the GH receptor.

Growth hormone (GH)[1] was identified as a growth factor more than 60 years ago, yet its mechanism of action is poorly understood (Greep, 1974). Recently, the receptors for a number of growth-promoting peptides have been shown to undergo ligand-activated tyrosyl phosphorylation [Cohen et al., 1980; Kasuga et al., 1982a; Roth & Cassell, 1983; Pang et al., 1985a; Nishimura et al., 1982; Frackelton et al., 1984; Jacobs et al., 1983; Rubin et al., 1983; Petruzzeli et al., 1984; Huang & Huang, 1986; for a review, see Carter-Su and Pratt (1984)]. As a first step toward determining whether the GH receptor is a ligand-activated tyrosine kinase, we investigated whether the GH receptor undergoes tyrosyl phosphorylation and whether GH stimulates the phosphorylation of its receptor in 3T3-F442A cells. This cell line undergoes GH-promoted differentiation from a fibroblast to an adipocyte form (Morikawa et al., 1982; Nixon & Green, 1984). Additionally, GH alters carbohydrate and lipid metabolism in the adipocyte form of this cell line (Schwartz, 1984; Schwartz et al., 1985). In the present study, we used cross-linking agents in combination with a highly specific antibody to phosphorylated tyrosyl residues (Pang et al., 1985a) to demonstrate that GH promotes tyrosyl phosphorylation of its receptor in 3T3-F442A fibroblasts and adipocytes.

Experimental Procedures

Materials. 3T3-F442A fibroblasts were kindly provided by Dr. H. Green, Harvard University. Recombinant DNA derived 22 000-dalton methionyl-hGH was a gift of Dr. A. Johanson, Genentech, Inc., and recombinant DNA derived 22 000-dalton hGH was provided by Eli Lilly. Protein A–Sepharose, aprotinin, leupeptin, and molecular weight standards were purchased from Sigma. Bovine serum albumin (BSA) was purchased from Sigma (fatty acid poor) or Armour (CRG-7). Dulbecco's modified Eagle's medium (DMEM), serum, antibiotics, and antimycotics were purchased from Grand Island Biological Co., Hyclone, or Irvine Scientific. Phosphate-free DMEM containing 25 mM HEPES was purchased from Irvine Scientific and Joklik's medium from Grand Island Biological Co. Sodium [^{125}I]iodide (16–17 mCi/μg of I) was purchased from Amersham. ^{32}P-Labeled orthophosphoric acid was purchased from New England Nu-

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[1] Abbreviations: GH, growth hormone; hGH, human growth hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; KRP, Krebs–Ringer phosphate buffer; PNPP, *p*-nitrophenyl phosphate; PMSF, phenylmethanesulfonyl fluoride; IGF-I, insulin-like growth factor I; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEA, triethylamine; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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clear. Disuccinimidyl suberate was obtained from Pierce and trypsin (TPCK) from Worthington. Dexamethasone was kindly provided by Merck, Inc., and porcine insulin was a gift of Eli Lilly Co. Rabbit serum containing anti-hGH antibody (C11981A) was obtained from the National Hormone and Pituitary Program. The remaining chemicals were of reagent grade.

Cell Culture. 3T3-F442A fibroblasts were disaggregated in trypsin (0.1% in phosphate-buffered saline, pH 7.0) and plated at a density of 200 cells/cm^2 in 100-mm dishes. Cells were grown to confluence and maintained in DMEM containing 1 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL Fungizone, and 10% calf serum at 37 °C in a humidified atmosphere of 10% CO_2–90% air. Cells were used 7–21 days after plating. Cell viability, by trypan blue exclusion, was always 95% or greater when tested. Spontaneous differentiation into adipocytes, assessed by phase-contrast microscopy, was less than 10% for all studies except when the adipocyte form of 3T3-F442A cells was studied. In the case of the adipocytes, confluent monolayers of cells were treated for 48 h with DMEM containing 10% fetal calf serum, 1 μg/mL insulin, 0.5 mM methylisobutylxanthine, and 0.25 μM dexamethasone. Monolayers were then maintained for 5–7 days with DMEM containing 10% fetal calf serum and used when 70–80% of cells demonstrated characteristics of adipocytes as assessed by phase-contrast microscopy.

^{125}I-GH Labeling of 3T3-F442A Fibroblasts. Human GH was labeled with Na^{125}I by Dr. C. Cameron (The University of Michigan) using the method of Thorell and Johanson (1971) to a specific activity of 76–172 μCi/μg. For all the experiments shown, recombinant DNA derived hGH was used. In a few early experiments, we used pituitary-derived GH (type A, lots K013183 and K120583), kindly provided by Dr. J. L. Kostyo (The University of Michigan), who isolated and purified the GH by ion-exchange chromatography on DEAE-cellulose according to Mills et al. (1969). Human GH prepared by this procedure had an average activity of 2.0 IU/mg. The different GH preparations used in this study showed no significant difference in binding ability or biological activity in cultured 3T3-F442A cells (Schwartz & Foster, 1986; data not shown). Because GH from pituitary sources gave similar results to those obtained by using recombinant DNA derived GH, we included experiments using either source when averaging results. Unless noted otherwise, cells in monolayer culture (100-mm dishes) were incubated in serum-free DMEM containing 1–2% BSA for 16–24 h. The cells were then washed twice in Krebs–Ringer phosphate buffer, pH 7.4 (KRP), containing 1% BSA and incubated in KRP–1% BSA containing ^{125}I-hGH (4 × 10^6 cpm/mL, 12–30 ng/mL, 3 mL/dish) at 24 °C for the indicated times. To evaluate nonspecific binding, 5 μg/mL unlabeled hGH was added to some dishes. The cells were then washed twice in ice-cold KRP and incubated with 0.4 mM disuccinimidyl suberate in KRP for 15 min at 4 °C. The buffer was removed and replaced with 3 mL of ice-cold buffer S (25 mM HEPES, 4 mM EDTA, 100 mM NaF, 10 mM $Na_2P_2O_7$, 0.85 mM Na_3VO_5, 100 μg/mL aprotinin, 100 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride, and 0.1% Triton X-100, pH 7.6). The dishes were scraped, and the cell preparation was centrifuged at 200000*g* for 60 min at 4 °C. Radioactivity in the solubilized fraction (supernatant) was determined by auto-γ counting.

Binding of ^{125}I-GH to Intact 3T3-F442A Fibroblasts. Cultured 3T3-F442A fibroblasts were incubated in serum-free medium for 24 h. Suspensions of fibroblasts were prepared by using Joklik's medium in the presence of 2 mM EDTA, as described by Deutsch et al. (1982), and resuspended at room temperature in KRP–5% BSA containing 0.5 mg/mL bacitracin. ^{125}I-GH at the designated concentration was added to 200 000 cells in 250 μL, and the mixture was shaken for the designated period of time. To evaluate nonspecific binding, 5 μg/mL unlabeled hGH was added to some of the cells. Binding was terminated by addition of 2 mL of ice-cold KRP. Bound hormone was separated from free hormone by rapid filtration through 0.45-μm filters (Millipore) which had been soaked in KRP–1% BSA for at least 30 min prior to assay. The filters were immediately washed twice with 2 mL of ice-cold KRP. ^{125}I collected on the filters was determined by using auto-γ counting. Samples were assayed in triplicate. The error bars indicate the standard error of the triplicate determinations. Where no error bars are visible, the magnitude of the standard error was less than the size of the symbol.

[^{32}P]P_i Incorporation into 3T3-F422A Fibroblasts. 3T3-F442A fibroblasts in monolayer culture were incubated in DMEM containing 2% BSA for 6–12 h at 37 °C. The cells were then incubated with [^{32}P]P_i (0.2 mCi/mL) in 3 mL of phosphate-free DMEM containing 0.1% BSA for 8–15 h at 37 °C under an atmosphere of 10% CO_2–90% air. The cells were washed twice with KRP containing 0.1% BSA and incubated for an additional 1 h at 24 °C with either control vehicle or 29–2200 ng/mL hGH. For the experiment depicted in Figure 5, GH was added directly to the ^{32}P-containing buffer for 1 h. The latter modification did not alter the ability of GH to stimulate phosphorylation of cellular proteins. The medium was replaced with ice-cold buffer S. Cells were scraped from the dishes at 4 °C and centrifuged as described above.

Binding to the Phosphotyrosyl Binding Antibody. The rabbit-derived phosphotyrosyl binding antibody was produced and purified as described previously (Pang et al., 1985a). Antibody (200 μg) was applied at 4 °C to protein A–Sepharose (200-μL packed volume) in a plastic disposable column. The column was equilibrated with antibody for at least 45 min and then washed with at least 20 volumes of 50 mM HEPES–0.1% Triton X-100, pH 7.6 (HT buffer). Samples containing solubilized proteins either cross-linked to ^{125}I-hGH or labeled with ^{32}P were passed through the column at 4 °C. The column was washed with at least 20 volumes of 150 mM NaCl, 50 mM HEPES, 100 μg/mL leupeptin, 100 μg/mL aprotinin, and 0.1% Triton X-100 (pH 7.6), followed by at least 20 volumes of HT buffer containing 100 μg/mL leupeptin and 100 μg/mL aprotinin. The column was eluted with 10 mM *p*-nitrophenyl phosphate (PNPP) in HT buffer containing 100 μg/mL leupeptin, 100 μg/mL aprotinin, and 1 mM PMSF. Radioactivity in each eluted sample was determined. Samples were stored on ice and analyzed by SDS–PAGE as rapidly as possible (generally within 45 min) following elution from the column to minimize proteolytic degradation.

Binding to Anti-GH Antibody. [^{32}P]P_i-labeled cells were treated with or without hGH, solubilized, and eluted from the phosphotyrosyl binding antibody column as described above. The column eluate was treated for 2 h at 4 °C with either rabbit serum containing anti-GH antibody or nonimmune rabbit serum, both at a dilution of 1:20 000. Additional unlabeled hGH (1 μg/mL) was added in some experiments. The eluates were then treated with protein A–Sepharose for an additional 30 min. Following centrifugation at 500*g* for 5 min, the pellet was washed twice with HT buffer and then boiled in sodium dodecyl sulfate (SDS) buffer. The protein A–Se-

pharose was removed by centrifugation (microfuge, 5 s) and the supernatant subjected to polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels in the presence of 0.1% SDS were prepared for electrophoresis according to the method of Laemmli (1970). Samples were diluted in SDS sample buffer (containing 1% SDS) with 40 mM dithiothreitol or 360 mM β-mercaptoethanol and boiled for 1 min before being loaded on gel lanes. Samples labeled with [^{32}P]P_i were treated with 70% (w/v) trichloroacetic acid at 4 °C for 15 min in the presence of insulin (100 μg) as a carrier. Samples were centrifuged in an Eppendorf microfuge for 10 min at 4 °C, and the precipitates were resuspended in 0.1 N NaOH to bring the pH to 7.0 prior to dilution in SDS sample buffer. Gels were fixed and stained in 7% glacial acetic acid, 25% isopropyl alcohol, and 0.1% Coomassie Brilliant Blue R and destained in 7% glacial acetic acid and 5% isopropyl alcohol. Gels were air-dried between two sheets of cellophane. Autoradiography was performed by using Kodak X-Omat XAR-5 film and Dupont Cronex Lightning Plus enhancing screens. Molecular weight standards included myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 400), BSA (M_r 66 000), catalase (M_r 58 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 29 000). Densitometry was performed with a Bio-Med Instruments laser scanning densitometer attached to an Apple IIE computer (Bio-Med Instruments videophoresis II data analysis computer program).

Phosphoamino Acid Analysis. Monolayers of fibroblasts were prepared and incubated with [^{32}P]P_i followed by GH for 1 h. Cellular proteins were solubilized, passed over the phosphotyrosyl binding antibody column as described above, and either analyzed directly by SDS–PAGE or first treated for 2 h at 4 °C with rabbit serum containing antibody to hGH. The resulting antibody complex was collected on protein A–Sepharose and subjected to SDS–PAGE as described above. The area of the gel containing ^{32}P-labeled protein with an approximate molecular weight of 114 000 (as indicated by autoradiography) was excised and stirred overnight with 20 mL of 20% methanol at 37 °C. After removal of the cellophane, the gel was cut in small pieces and dried at 70 °C. The pieces of dried gel were either digested with trypsin and subjected to limited acid hydrolysis (procedure A) or subjected to extensive base hydrolysis (procedure B).

For procedure A, pieces of dried gel containing ^{32}P-labeled protein were incubated with 1 mL of a freshly prepared solution of 50 mM NH_4HCO_3 (pH 8) to which was added 100 μL of 1 mg/mL trypsin. The mixture was stirred for 6 h at 37 °C, whereupon another 100 μL of 1 mg/mL trypsin was added to the mixture. Tryptic digestion was continued with stirring for 12 h more at 37 °C. After removal of insoluble material from the digestion mixture by centrifugation, the digestion mixture was lyophilized. The lyophilisate was hydrolyzed in contact with vapor from 6 N HCl in an evacuated sealed chamber at 110 °C for 2 h. The resulting digest was taken up 3 times in 50 μL of water and evaporated to dryness. This residue was taken up in 5 μL of electrophoretic buffer [acetic acid–pyridine–water (50:5:945 v/v) and 5 mM EDTA, pH 3.5] containing carrier *O*-phosphotyrosine, *O*-phosphothreonine, and *O*-phosphoserine (each at 1 mM) and subjected to thin-layer electrophoresis on 10 × 20 cm Whatman K2 cellulose plates at 100 V/cm for 25 min at 12 °C. The thin-layer plates were sprayed with ninhydrin to delineate the migration positions of the phosphoamino acids and then subjected to autoradiography.

For procedure B, pieces of dried gel containing ^{32}P-labeled protein were placed in a 7 × 11 mm Teflon tube (Wilmad WG-1264) containing 10 nmol of *O*-phosphotyrosine in 0.2 mL of 5 N KOH. The Teflon tube was then placed in a 0.3-mL Reacti-vial (Pierce) containing 0.15 mL of 5 N KOH. The Reacti-vial was sealed with a Teflon-lined screw cap and heated for 70 min at 155 °C in a Reacti-bath (Pierce). The 0.15 mL of 5 N KOH surrounding the hydrolysis tube prevented evaporation of liquid from the open tube within the Reacti-vial. After cooling, the 0.2 mL of hydrolysate was transferred with 0.8 mL of H_2O to a polyethylene tube containing 1 g of Dowex 50W X-12 (acid form) and stirred for 30 min. The solution was removed by filtration, and the Dowex resin was rinsed twice with 0.5 mL of H_2O. The Dowex was then transferred back to a polyethylene tube and shaken with 2 mL of 5 N NH_3 for 2 h. The supernatant solution was collected by filtration and taken to dryness under reduced pressure. The residue was taken up and reevaporated 3 times with 0.1 mL of H_2O and 2 times with 25 μL of 95% EtOH–TEA–H_2O (4:4:2). The resulting residue was taken up in 25 μL of 95% EtOH–TEA–H_2O (6:2:2) and reacted with 25 μL of 10% phenyl isothiocyanate in 95% EtOH. After 15 min at 50 °C, the reaction mixture was evaporated to dryness and taken up in 9:91 acetonitrile–0.07 M phosphoric acid that had been neutralized to pH 6.8 with TEA. The resulting solution was subjected to HPLC on a C_{18} column according to the procedure of Pang et al. (1984) for separation of the *N*-phenylthiocarbamyl phosphoamino acid derivatives. One-milliliter fractions were collected from the HPLC column and evaporated to dryness with a Savant SpeedVac concentrator. The residue from each fraction was transferred with water to small plastic cups made from caps from 0.5-mL microfuge tubes (Sarstedt 72.699). The cups were fixed with rubber cement to a piece of paper and placed overnight in a hood to evaporate the liquid. The residues from each of the fractions from the HPLC column in the cups affixed to the paper were subjected to autoradiography to localize the elution position of ^{32}P-labeled material.

Statistics. For averaged data, means ± SE are given.

Results

Interaction of GH Receptor–GH Complexes with Phosphotyrosine Binding Antibody. To determine whether GH receptor–GH complexes contained phosphotyrosyl residues, the ability of GH receptors, specifically cross-linked to ^{125}I-GH, to bind to the phosphotyrosyl binding antibody was assessed. 3T3-F442A fibroblasts were incubated with ^{125}I-GH in the absence and presence of excess unlabeled GH. Complexes of GH and its receptor were stabilized by the chemical cross-linking agent disuccinimidyl suberate, as described previously (Carter-Su et al., 1984), and solubilized in a buffer containing Triton X-100. Solubilized proteins were immunoadsorbed to phosphotyrosyl binding antibody immobilized on protein A–Sepharose. After extensive washing to remove nonspecifically bound proteins, specifically bound proteins were eluted with the hapten PNPP. Figure 1 compares the cross-linked proteins before and after adsorption to the phosphotyrosyl binding antibody. SDS–PAGE revealed that before immunoadsorption, the solubilized proteins contained a major ^{125}I-labeled species of M_r 134 000 ± 1000, n = 19 (Figure 1, lane A), not seen when cells were incubated with excess unlabeled GH (Figure 1, lane B). The ability of excess unlabeled GH (but not prolactin) to eliminate radiochemical labeling of the M_r 134 000 species is consistent with the notion that this material (Figure 1, lane A) reflects a specific interaction between GH and its receptor. This complex has a molecular weight similar

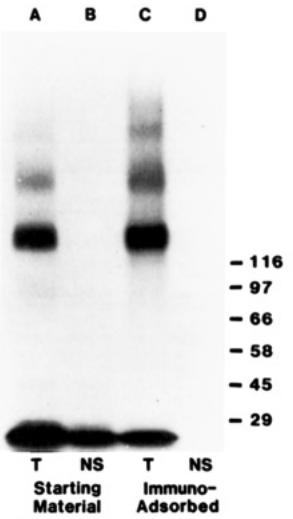

FIGURE 1: Binding of ^{125}I-GH affinity-labeled GH receptor to the phosphotyrosyl binding antibody. 3T3-F442A fibroblasts were incubated for 1 h at 24 °C in ^{125}I-hGH (4×10^6 cpm/mL, 27 ng/mL) alone (T, lanes A and C) or in combination with 5 μg/mL unlabeled hGH (NS, lanes B and D) (three dishes per condition). Cells were washed, and disuccinimidyl suberate (0.4 mM) was added. Following solubilization in ice-cold buffer S containing 0.1% Triton X-100, the solubilized material was added to a phosphotyrosyl binding antibody column as described under Experimental Procedures. Bound proteins were eluted in 10 mM PNPP (200-μL void volume followed by three aliquots of 400 μL). Solubilized proteins before chromatography (aliquots of 80 μL, representing approximately 1% of total starting material) were prepared in β-mercaptoethanol and applied to the SDS–polyacrylamide gel (lanes A and B). Column eluates (160 μL of the first 400-μL aliquot, representing approximately 25% of eluted radioactivity) were prepared in the presence of β-mercaptoethanol and applied to the gel (lanes C and D). Molecular weights ($\times 10^{-3}$) of the protein standards are indicated on the right of the panel. Refer to Figure 2 for the approximate migration of the 205 000-dalton marker.

to those reported for the cross-linked GH receptor of rat adipocytes, rat hepatocytes, and human IM-9 lymphocytes (Carter-Su et al., 1984; Donner, 1983; Asakawa et al., 1985; Hughes et al., 1983). The higher molecular weight radiochemically labeled bands [M_r 255 000 ± 4000 (n = 18), 375 000 ± 12 000 (n = 16), and 494 000 ± 21 000 (n = 9)] in Figure 1 were obtained with variable yields and are ascribed to cross-linked aggregates of the receptor hormone complex. High molecular weight ^{125}I-GH–receptor complexes have been noted previously in preparations of cross-linked complexes of GH and its receptors from rat adipocytes and hepatocytes (Carter-Su et al., 1984; Donner, 1983; Gorin & Goodman, 1984). SDS–PAGE also revealed the presence of an ^{125}I-labeled protein of M_r 22 000. This material is ascribed to nonspecifically bound cell-associated GH (not complexed with its receptor) as well as receptor-bound GH which escaped cross-linking.

SDS–PAGE after immunoadsorption and elution of solubilized cellular proteins on immobilized phosphotyrosyl binding antibody also revealed the presence of an ^{125}I-protein complex of M_r 134 000 (Figure 1, lane C) not seen when cells were treated with additional excess unlabeled GH (Figure 1, lane D). Control experiments where ^{125}I-GH which had not been incubated with cells was subjected to the same immunoadsorption and elution procedures using immobilized antibody showed that the amount of ^{125}I-GH recovered in the eluate was only 1.2% of that seen with the cell extracts. This result indicates that ^{125}I-GH by itself does not bind to the immobilized antibody. Thus, it is unlikely that the binding of the receptor complexes to the immobilized antibody is the result of a direct interaction of receptor-bound GH and the antibody.

It is important to note that the presence of excess unlabeled GH resulted in the loss of the M_r 22 000 ^{125}I-GH species in the PNPP eluate from the antibody column (Figure 1, lane D). The most plausible explanation of this observation is that nonspecifically bound cell-associated ^{125}I-GH did not bind to the antibody column, whereas both the cross-linked and non-cross-linked GH–receptor complexes bound to the antibody column and appeared in the PNPP eluate. Thus, the appearance of the M_r 22 000 band corresponding to ^{125}I-GH (Figure 1, lane C) is assumed to reflect the dissociation of ^{125}I-GH from the non-cross-linked receptor complex.

Densitometric analysis of the radioactive bands (M_r 22 000, 134 000, 255 000, and 375 000) obtained upon SDS–PAGE of the solubilized cellular proteins indicated values of 35% and 45% for the fraction of specifically bound ^{125}I-GH that was cross-linked to its receptor for samples analyzed before and after immunoadsorption, respectively. Comparison of the radioactivity in the species corresponding to cross-linked receptor (M_r 134 000, 255 000, and 375 000) obtained before and after immunoadsorption indicated that between 3% and 8% of the cross-linked complex was recovered from the antibody column by using the protocol described in Figure 1. The observation that more complex (9–17%) was recovered when the incubation with the phosphotyrosyl binding antibody was allowed to proceed overnight suggests the low extent of recovery of GH receptors from the antibody column was due in part to incomplete binding of the cross-linked phosphorylated receptor to the immobilized antibody.

One could argue that the radiochemically labeled material recovered from the antibody column was nonspecifically adsorbed to the antibody or interacted with the antibody via phosphorylated seryl or threonyl residues. To demonstrate that the radioactivity bound to and eluted from the column reflects a specific interaction of ^{125}I-labeled GH receptor with the phosphotyrosine binding antibody, the effect of phosphorylated amino acids on the interaction between the antibody and the receptor complex was tested. 3T3-F442A fibroblasts were cross-linked to ^{125}I-GH with disuccinimidyl suberate, solubilized, and centrifuged. Aliquots of the supernatant were treated with vehicle or brought to a final concentration of 2 mM with *O*-phosphotyrosine, *O*-phosphoserine, or *O*-phosphothreonine. The resulting solutions were passed over the phosphotyrosyl binding antibody column. The presence of *O*-phosphotyrosine eliminated ^{125}I-GH affinity-labeled proteins from the eluate of the phosphotyrosyl binding antibody column, reducing radioactivity (cpm in the eluates) recovered in the eluate by 95% ± 5% (n = 3), consistent with the notion that the immobilized antibody bound phosphotyrosyl residues in the ^{125}I-GH–receptor complex. In contrast, the presence of *O*-phosphoserine and *O*-phosphothreonine caused only a small reduction (8% ± 16% and 17% ± 1%, respectively, n = 3) in the amount of radioactivity in the PNPP eluate from the antibody column. SDS–PAGE of the PNPP eluates from the antibody column revealed the presence of the major ^{125}I-labeled M_r 134 000 complex in all samples except the sample that had been treated with *O*-phosphotyrosine prior to immunoadsorption (Figure 2). The fact that samples treated with *O*-phosphotyrosine (Figure 2, lane C) failed to show any M_r 134 000 species, any higher molecular weight forms of the cross-linked receptor, or a M_r 22 000 band corresponding to ^{125}I-GH suggests the presence of at least one phosphotyrosyl residue in the GH–receptor complexes.

Cross-linked ^{125}I-GH–receptor complexes also bound to the phosphotyrosyl binding antibody when the differentiated adipocyte form of 3T3-F442A cells was used in place of the

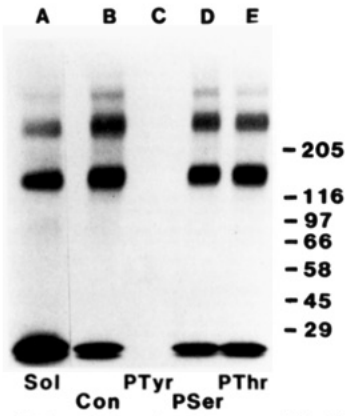

FIGURE 2: Specificity of the phosphotyrosyl binding antibody for phosphotyrosyl residues in GH receptor–^{125}I-GH complexes. 3T3-F442A fibroblasts (12 dishes) were incubated with ^{125}I-hGH (4 × 10^6 cpm/mL, 15 ng/mL) for 1 h at 24 °C and cross-linked with disuccinimidyl suberate. Eighty microliters of solubilized cell extract was prepared and applied to lane A. Prior to exposure to the immobilized phosphotyrosyl binding antibody, solubilized cell extracts were divided into four equal aliquots of 9 mL and treated with control vehicle (lane B) or made 2 mM in *O*-phosphotyrosine (lane C), *O*-phosphoserine (lane D), or *O*-phosphothreonine (lane E). After each sample was passed over the anti-phosphotyrosine antibody column, the column was eluted in 10 mM PNPP (one aliquot of 200 μL followed by three aliquots of 400 μL). In each case, the second aliquot contained peak radioactivity, and equal volumes (160 μL) of each sample were analyzed in the presence of β-mercaptoethanol on a 3–10% SDS–polyacrylamide gel. The migration position of the molecular weight standards (×10^{-3}) is indicated on the right.

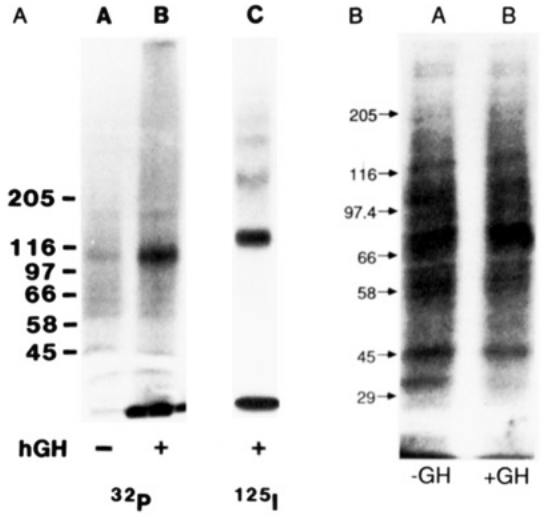

FIGURE 3: Incorporation of [^{32}P]P_i into the GH receptor. (Panel A) 3T3-F442A fibroblasts (4 dishes/condition) were incubated overnight with [^{32}P]P_i (0.2 mCi/mL) in phosphate-free DMEM at 37 °C followed by control vehicle (lane A) or 29 ng/mL hGH (lane B) in KRP + 0.1% BSA for an additional 1 h at 24 °C. Solubilized cell extracts were immediately passed over the phosphotyrosyl binding antibody column. Samples were eluted with 10 mM PNPP as in Figure 1. In a parallel experiment, 3T3-F442A fibroblasts were incubated for 1 h with ^{125}I-GH (4 × 10^6 cpm/mL, 29 ng/mL, lane C), cross-linked with disuccinimidyl suberate, solubilized, and passed over the phosphotyrosyl binding antibody column as in Figure 1. Proteins in the eluate were concentrated by trichloroacetic acid precipitation and analyzed by SDS–PAGE on a 3–10% polyacrylamide gel in the presence of β-mercaptoethanol. Lane C contains one-fifth the amount of eluate contained in lanes A and B. (Panel B) 3T3-F442A fibroblasts were incubated for 4 h with [^{32}P]P_i (0.125 mCi/mL) followed by vehicle (lane A) or 2200 ng/mL hGH (lane B) in KRP–0.1% BSA for an additional 1 h at 24 °C. Portions (5 μL) of the solubilized cell extracts were analyzed by SDS–PAGE in the presence of β-mercaptoethanol. Similar results (i.e., lack of increase in overall cellular phosphorylation due to GH) were observed whether the cells were incubated for 4 or 15 h with [^{32}P]P_i, with 0.125 or 0.2 mCi/mL [^{32}P]P_i, or with 2200 or 30 ng/mL GH.

fibroblast form. This indicates that complexes of GH and its receptor in the adipocytes interact with the phosphotyrosyl binding antibody as do GH–receptor complexes in the fibroblast.

Incorporation of [^{32}P]P_i into the GH Receptor. To determine whether the GH receptor in 3T3-F442A fibroblasts is phosphorylated in response to GH, cells were incubated overnight with [^{32}P]P_i at 37 °C in phosphate-free DMEM containing 0.1% BSA. Following exposure to [^{32}P]P_i, cells were exposed to either 0 or 29 ng/mL hGH for an additional 1 h. Medium containing the GH was removed, and the cells were immediately solubilized and applied to the antibody column. SDS–PAGE analysis of eluted proteins revealed the presence of a ^{32}P-labeled protein with a mobility somewhat less [M_r 114 000 ± 2000 (n = 8)] than that of the ^{125}I-GH–receptor complex [M_r 134 000 ± 1000 (n = 19)] (compare lanes A and C, Figure 3A) as would be expected for GH–receptor which had not been cross-linked to GH (expected M_r 112 000). The amount of this phosphoprotein was reduced [(7 ± 1)-fold, n = 6, as determined by densitometry] when the cells were not treated with GH prior to solubilization (compare lanes A and B, Figure 3A). No phosphorylated protein of M_r 22 000 was seen with either condition, consistent with the notion that GH is not phosphorylated at tyrosyl residues under the incubation conditions used. The GH-induced increase in ^{32}P labeling of the 114 000-dalton protein is unlikely to be due to a change in the ATP pool specific activity, since GH does not increase overall labeling of cellular proteins (Figure 3B).[2]

Anti-GH antibodies were used to provide additional evidence that the 114 000-dalton ^{32}P-labeled species is the GH receptor. Cells were incubated with [^{32}P]P_i followed by GH. Solubilized cellular proteins were then immunoadsorbed and eluted from the phosphotyrosyl binding antibody column. The eluates were treated with (a) rabbit serum containing anti-GH antibody, (b) nonimmune rabbit serum, or (c) serum containing anti-GH antibody as well as unlabeled GH (1 μg/mL). Antibody–antigen complexes were adsorbed on protein A–Sepharose, solubilized with SDS, and subjected to SDS–PAGE. The major phosphorylated species isolated with the anti-GH antibody had a molecular weight of 114 000 (Figure 4, lanes A and E). This protein was absent when nonimmune serum was used (Figure 4, lanes B and F) and was markedly reduced when anti-GH complexes formed in the presence of excess unlabeled GH (Figure 4, lane G). Furthermore, no detectable M_r 114 000 phosphoprotein was immunoprecipitated by the anti-GH antibody from extracts of ^{32}P-labeled cells not exposed to GH (Figure 5).

Other ^{32}P-labeled bands appeared in the eluates of the phosphotyrosine binding antibody column (see Figure 3A, lanes A and B). These were not immunoadsorbed by anti-GH but remained in the supernatant solutions after precipitation of the immune complexes by protein A–Sepharose (Figure 4, lanes C and D). As expected from the selective immunoprecipitation of the M_r 114 000 protein by anti-GH antibody, the intensity of the M_r 114 000 band was decreased in the supernatant solution from the eluate treated with anti-GH antibody (Figure 4, lane C) compared to the supernatant solution from eluate treated with nonimmune serum (Figure 4, lane D). An average of 29% (estimated by densitometric analysis, n = 4) of the M_r 114 000 phosphoprotein present in the eluate of the phosphotyrosyl binding antibody column was

[2] The high basal level of phosphorylation in samples which had not been subjected to immunoadsorption and elution precluded detection of GH-stimulated ^{32}P incorporation into protein (Figure 3B).

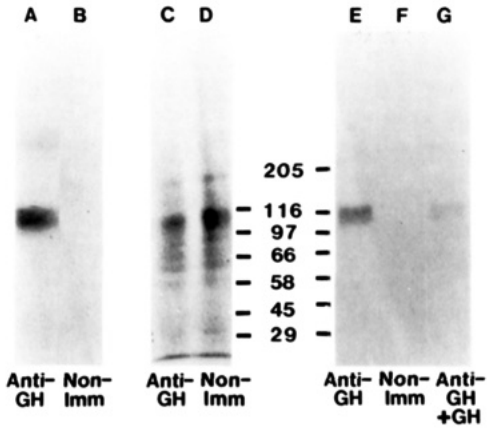

FIGURE 4: Adsorption of phosphoproteins by anti-GH antibody. 3T3-F442A fibroblasts were treated with [^{32}P]P_i followed by 50 ng/mL hGH and adsorbed to the phosphotyrosyl binding antibody column. Column eluates were treated with a 1:20000 dilution of rabbit serum containing anti-hGH antibody (lanes A, C, and E), a 1:20000 dilution of nonimmune rabbit serum (lanes B, D, and F), or a 1:20000 dilution of rabbit serum containing anti-hGH antibody and 1 μg/mL hGH (lane G) for 2 h at 4 °C. The immunoglobulin-bound proteins were then adsorbed by protein A–Sepharose for 30 min at 4 °C and centrifuged. The supernatants were removed, and the adsorbed proteins were released by boiling 1 min in an equal volume of SDS buffer in the presence of β-mercaptoethanol. Aliquots of released protein (200 μL) were applied to a 3–10% SDS–polyacrylamide gel (lanes A, B, and E–G). Aliquots of supernatants (100 μL) were combined with 100 μL of SDS buffer including β-mercaptoethanol before electrophoresis (lanes C and D). In this experiment, the M_r 114000 band migrated as a doublet. This may be related to proteolysis; however, the significance of this finding is not known.

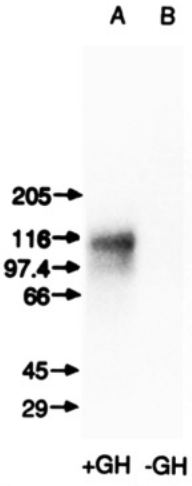

FIGURE 5: Effect of prior exposure of cells to GH on adsorption of phosphoproteins by anti-GH antibody. 3T3-F442A fibroblasts were incubated with [^{32}P]P_i overnight and then for an additional 1 h with either 30 ng/mL hGH (lane A) or vehicle (lane B) added directly to the medium. Proteins were solubilized and incubated for 1 h with immobilized phosphotyrosyl binding antibody. Proteins were eluted from the antibody and treated with a 1:20000 dilution of rabbit serum containing anti-hGH antibody as described for Figure 4. The immunoprecipitated proteins were applied to a 3–10% SDS–polyacrylamide gel. In this experiment, a fainter second band was seen migrating below the M_r 114000 phosphoprotein. This band is thought to be related to proteolysis and has not been seen in other experiments (compare to Figure 4).

precipitated by the anti-GH antibody. The ability of anti-GH to bind the ^{32}P-labeled protein indicates that at least 29% of the M_r 114000 band is phosphorylated GH receptor from which GH dissociated during SDS–PAGE. Control experiments indicated that incomplete immunoprecipitation was partially or wholly responsible for the M_r 114000 ^{32}P-labeled material present in the supernatant solution after treatment

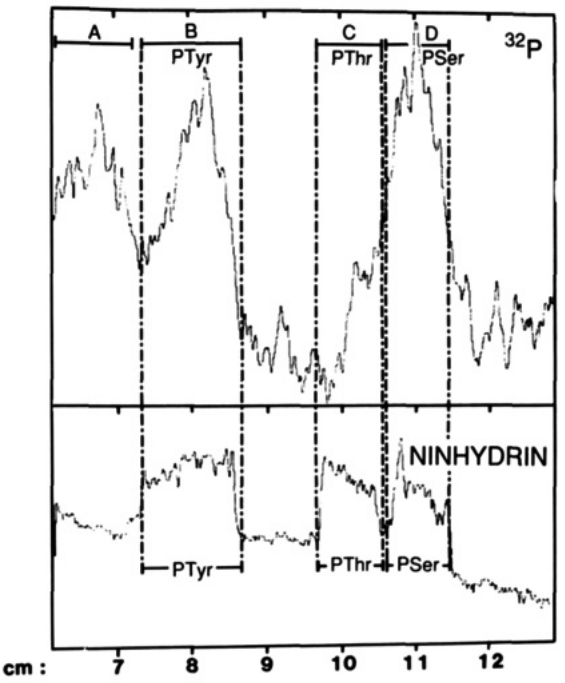

FIGURE 6: Analysis of phosphoamino acids of the GH receptor. 3T3-F442A fibroblasts were treated with [^{32}P]P_i followed by 2200 ng/mL hGH and adsorbed to the phosphotyrosyl binding antibody column as described in Figure 4. Eluted proteins were concentrated by trichloroacetic acid precipitation and analyzed on a 3–10% SDS–polyacrylamide gel. The area of the gel containing ^{32}P-labeled protein with an approximate molecular weight of 114000 was excised and analyzed for phosphoamino acid content by procedure A as described under Experimental Procedures. The x axis represents the migration distance in centimeters from the origin in the thin-layer cellulose plate used to separate *O*-phosphoserine (PSer), *O*-phosphothreonine (PThr), and *O*-phosphotyrosine (PTyr). The lower panel is a densitometric scan of the ninhydrin-stained unlabeled *O*-phosphoamino acid standards. The upper panel is a densitometric scan of the autoradiograph of the partial acid hydrolysate of the ^{32}P-labeled M_r 114000 protein. In the upper panel, the migration of incompletely digested phosphorylated peptides is denoted as peak A, the migration of phosphotyrosine as peak B, the migration of phosphothreonine as peak C, and the migration of phosphoserine as peak D. The dashed lines delineate the boundaries of the amino acid standards.

with anti-GH antibody (Figure 4, lane C).

To confirm that phosphotyrosine was present in the GH receptor, we analyzed the phosphoamino acid content of the M_r 114000 phosphoprotein. Cells were treated with [^{32}P]P_i followed by 100 nM (2200 ng/mL) GH. Solubilized cellular proteins were applied to the phosphotyrosyl binding antibody column. Eluted proteins were subjected to SDS–PAGE and autoradiographed. The portion of the gel containing the major phosphoprotein migrating with M_r 114000 was excised from the gel, rehydrated, digested with trypsin, and subjected to limited acid hydrolysis. The hydrolysis products were analyzed by thin-layer cellulose electrophoresis. Figure 6 is a densitometric scan of the ninhydrin-stained standards (lower panel) and of the autoradiograph (upper panel). A substantial portion of the [^{32}P]P_i incorporated into the GH receptor comigrated with *O*-phosphotyrosine (Figure 6, peak B). An equal or even greater amount of radioactivity comigrated with *O*-phosphoserine (Figure 6, peak D). Additional radioactive material was observed migrating with a mobility less than that of phosphotyrosine (Figure 6, peak A; data not shown). This material is believed to represent incompletely digested phosphorylated peptides.

To verify that the ^{32}P-labeled material migrating with *O*-phosphotyrosine was not an incompletely hydrolyzed phos-

phorylated peptide or some other phosphorylated material, and to provide further evidence that the material identified as *O*-phosphotyrosine arose from phosphorylated GH receptor, the eluate from the phosphotyrosyl binding antibody column (which putatively contains ^{32}P-labeled GH receptor complexed with hGH) was treated with rabbit antiserum to hGH. The resulting antibody complex was collected with protein A–Sepharose and subjected to SDS–PAGE. ^{32}P-Labeled material migrating with a molecular weight of 114000 was excised from the gel and hydrolyzed in 5 N KOH by a procedure which hydrolyzes proteins to their constituent amino acids (Martensen & Levine, 1983). (This hydrolytic procedure results in essentially complete elimination of phosphate from *O*-phosphoseryl and *O*-phosphothreonyl residues in proteins and about 70% recovery of *O*-phosphotyrosine.) The amino acids in the base hydrolysate were absorbed on Dowex-50 acid form, eluted with ammonia, converted to phenylthiocarbamyl derivatives, and subjected to HPLC according to the procedure of Pang et al. (1984) for separating phosphoamino acids. A single radiochemically labeled component was found after derivatization of the ammonia eluate from the Dowex-50 with phenyl isothiocyanate. This ^{32}P-labeled compound comigrated with *N*-phenylthiocarbamyl-*O*-phosphotyrosine during HPLC, suggesting that the GH receptor underwent tyrosyl phosphorylation.

Effect of Exposure Time and Concentration of GH on Tyrosyl Phosphorylation of the GH Receptor. Assuming that the GH receptor must be phosphorylated on at least one tyrosyl residue to bind phosphotyrosyl binding antibody, ^{125}I-GH can be used to monitor the dose–response and time course for formation of tyrosyl phosphorylated receptor. This approach circumvents the problems associated with metabolically labeling cells with the large amounts (up to 10 mCi) of [^{32}P]P_i required to visualize the phosphorylated receptor. To determine whether phosphorylation of the GH receptor parallels binding of GH to its receptor, 3T3-F442A fibroblasts were exposed to ^{125}I-GH in concentrations from 5 to 40 ng/mL for 1 h at room temperature. Increasing concentrations of ^{125}I-GH resulted in a dose-related increase in ^{125}I-GH specifically associated with the cells (Figure 7A, inset). A similar dose-related increase was observed when after binding and cross-linking ^{125}I-GH to cells, cell extracts were adsorbed and eluted from the phosphotyrosyl binding antibody column (Figure 7A). The yield of ^{125}I-GH–receptor complex binding to the phosphotyrosyl binding antibody was near-maximal when 20 ng/mL GH was incubated with the cells, a concentration of GH at which maximal or near-maximal GH binding to intact 3T3 fibroblasts was observed. The major ^{125}I-labeled species recovered after immunoadsorption, as determined by SDS–PAGE, was the 134000-dalton band. The intensity of the M_r 134000 band, as well as the higher molecular weight GH–receptor complexes, increased as a function of GH concentration in samples before immunoadsorption (Figure 7B, lanes A–D) and in samples eluted from the antibody column (Figure 7B, lanes E–H). The data depicted in the inset of Figure 7A indicate that half-maximal ^{125}I-GH binding to intact cells occurs at 7 ng/mL. The GH concentration resulting in half-maximal binding of ^{125}I-GH–receptor complexes to the phosphotyrosyl binding antibody, estimated both by cpm eluted from the column (Figure 7A) and by densitometric analysis of the autoradiogram (Figure 7B, lanes E–H), was approximately 6 ng/mL. Thus, increases in GH–receptor complexes containing phosphotyrosyl residues (eluted from the column) paralleled the total amount of ^{125}I-GH specifically bound to its receptor.

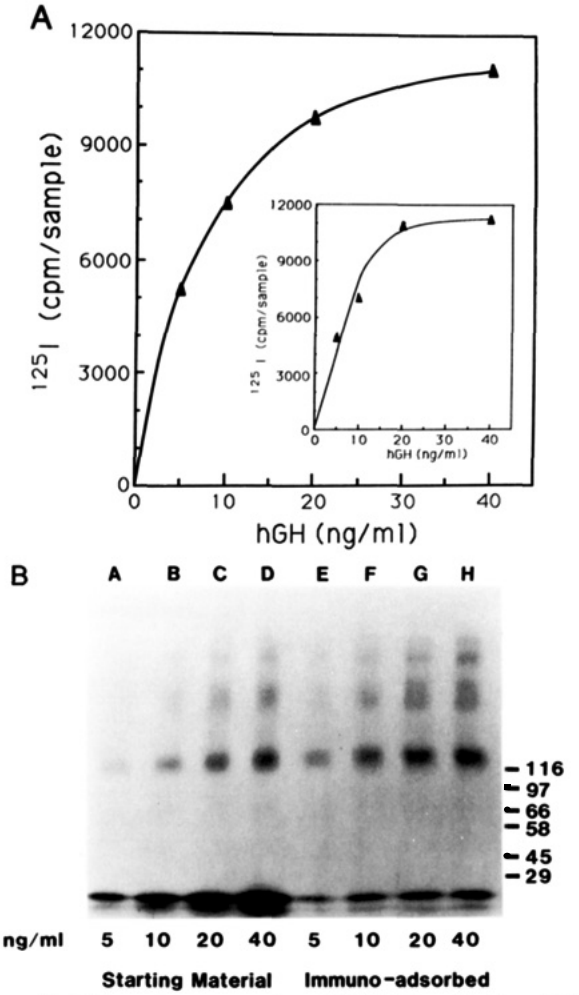

FIGURE 7: Effect of concentration of ^{125}I-GH on binding of GH receptors to the phosphotyrosyl binding antibody. 3T3-F442A fibroblasts (three dishes per condition) were incubated with ^{125}I-GH at the indicated concentrations for 1 h at 24 °C, cross-linked with disuccinimidyl suberate, solubilized, and passed over the antibody column as in Figure 1. In panel A, the radioactivity of the proteins eluted from the column was determined and plotted as a function of GH concentration. In panel B, equal volumes of the samples prior to immunoadsorption (80 μL) and each of the second aliquots of the antibody column (160 μL), containing peak radioactivity, were treated with β-mercaptoethanol and analyzed on a 3–10% SDS–polyacrylamide gel. (Lanes A–D) Extracts of solubilized cells before immunoadsorption. (Lanes E–H) Samples after elution from the phosphotyrosyl binding antibody column. (Panel A inset) 3T3-F442A fibroblasts (200000 cells/250 μL) were prepared as described under Experimental Procedures and incubated with ^{125}I-GH at the indicated concentrations for 1 h at 24 °C in the absence and presence of 1 μg/mL unlabeled hGH. Bound ^{125}I-GH was determined as described under Experimental Procedures. The results shown represent specifically bound radioactivity, determined by subtracting nonspecifically bound radioactivity from total radioactivity associated with the cells.

Similar studies (data not shown) indicated parallel time dependencies for GH-stimulated phosphorylation and GH binding to 3T3-F442A fibroblasts. Approximately 23 min was required for both half-maximal tyrosyl phosphorylation and half-maximal association of the GH with the cells at an ^{125}I-GH concentration of 21 ng/mL.

Discussion

GH Receptor Undergoes GH-Promoted Phosphorylation on Tyrosyl Residues. This study of the interaction of GH–GH receptor complexes with phosphotyrosyl binding antibodies and anti-GH antibodies together with phosphoamino acid analysis provides strong evidence that GH receptors undergo GH-promoted tyrosyl phosphorylation. SDS–PAGE indicated an

apparent molecular weight of 114 000 ± 2000 (n = 8) for the ^{32}P-labeled phosphorylated form of the receptor, whereas the apparent molecular weight of the cross-linked ^{125}I-GH–GH receptor complex was 134 000 ± 1000 (n = 19). The 2000-dalton difference in mean molecular weight between the receptor (112 000) cross-linked to ^{125}I-GH (22 000) and the phosphorylated un-cross-linked GH receptor (114 000) is consistent with our previously documented (Pang et al., 1984) increases in electrophoretic mobility of proteins upon cross-linking with disuccinimidyl suberate. Further evidence that this 114 000-dalton species is the GH receptor is provided by its ability to be immunoprecipitated by anti-hGH antibody only when cells had been incubated with GH prior to membrane solubilization. This observation indicates that the 114 000-dalton species, prior to denaturation in SDS buffer, exists as a complex with GH.

Although Baldwin et al. (1983) have demonstrated that tyrosyl residues in human GH are phosphorylated by epidermal growth factor receptor in A431 cell membranes (Baldwin et al., 1983), it is unlikely that GH receptor–^{125}I-GH complexes bound to the phosphotyrosyl binding antibody column via phosphotyrosyl residues in GH. ^{125}I-GH by itself did not bind to the anti-phosphotyrosine antibody. Moreover, no phosphorylation of GH could be detected upon incubation of GH with cells metabolically labeled with ^{32}P.

The observed dependence on the presence of ligand for GH receptor tyrosyl phosphorylation is similar to that observed for ligand-activated tyrosine kinases such as the receptors for insulin, IGF-I, epidermal growth factor, and platelet-derived growth factor [Cohen et al., 1980; Kasuga et al., 1982a; Roth & Cassell, 1983; Pang et al., 1985a; Nishimura et al., 1982; Frackelton et al., 1984; Jacobs et al., 1983; Rubin et al., 1983; Petruzzeli et al., 1984; see also a review of this subject by Carter-Su and Pratt (1984)]. Although we consider it most likely that the GH receptor itself is a ligand-activated tyrosine kinase, the alternative possibility, that upon binding GH the GH receptor becomes a substrate for another tyrosine kinase, has not been excluded. We also cannot rule out the possibility that GH binding to its receptor results in the activation of a cellular tyrosine kinase other than the GH receptor which in turn increases the number of phosphorylated tyrosyl residues on GH receptors.

The phosphotyrosyl binding antibody used in this study has also been used to isolate catalytically competent insulin receptor and characterize insulin-promoted tyrosyl phosphorylation of its receptor in FAO hepatocytes (Pang et al., 1985a,b). Similar antibodies have been used to study phosphorylated tyrosyl residues on platelet-derived growth factor receptors (Frackelton et al., 1984; Ek & Heldin, 1984), on bombesin receptors (Cirillo et al., 1986), and on cellular proteins other than the tyrosine kinases themselves (Ek & Heldin, 1984; Ross et al., 1981; Gacon et al., 1984; Maher et al., 1985; White et al., 1985). Use of an antibody that is highly specific for phosphotyrosyl residues in combination with affinity labeling of membrane receptors made it feasible to determine tyrosyl phosphorylation in the low-abundance GH receptor (8000–14 000 sites/3T3-F442A cell; Nixon & Green, 1983). In the case of the GH receptor, Asakawa et al. (1985) were unable to attribute tyrosine kinase activity to the GH receptor in IM-9 lymphocytes and rat liver membranes. In their study, GH receptor was partially purified by using immobilized wheat germ agglutinin and assayed for tyrosine kinase activity by using both the receptor and exogenous compounds as substrates. It was not clear to us why GH-promoted phosphorylation of its receptor should not also occur in these cell types. We therefore initiated studies of GH-promoted phosphorylation of its receptor in cultured IM-9 cells and cultured rat hepatocytes using our phosphotyrosine binding antibody. In preliminary studies, we have found that GH receptor–^{125}I-GH complexes from human IM-9 lymphocytes and from rat H35 cells bind to the phosphotyrosyl binding antibody, suggesting that GH promotes phosphorylation of its receptor on tyrosyl residues in these cells as well as in the 3T3-F442A fibroblasts and adipocytes.

GH Receptor May Be Phosphorylated on Seryl Residues as Well as Tyrosyl Residues. Phosphoamino acid analysis of the M_r 114 000 phosphoprotein eluted from the phosphotyrosyl binding antibody indicates the presence of phosphoseryl residues in addition to phosphotyrosyl residues. Since the antibody column appears to bind only proteins containing phosphotyrosyl residues, this finding suggests that at least some, if not most, of the M_r 114 000 phosphoproteins contain both phosphotyrosyl and phosphoseryl residues. The presence of phosphoseryl as well as phosphotyrosyl residues in the GH receptor would not be inconsistent with it being a ligand-activated tyrosine kinase. The other membrane receptor tyrosine kinases described to date, as well as most of the viral-transforming tyrosine kinases, are also phosphorylated on seryl, and often threonyl, residues (Carter-Su & Pratt, 1984; Frackelton et al., 1984; Pang et al., 1985b; Ek & Heldin, 1984; Hunter & Cooper, 1981; Kasuga et al., 1982b; Jacobs & Cuatrecasas, 1986; Hunter, 1982). Evidence has been presented suggesting that threonine phosphorylation of the epidermal growth factor receptor by protein kinase C (Lin et al., 1986; Davis & Czech, 1985; Friedmann et al., 1984; Cochet et al., 1984; Lee & Weinstein, 1979) and serine phosphorylation of insulin receptors (Pang et al., 1985b; Bollag et al., 1986) may play a regulatory role in the function of these receptors. The effects of cellular serine kinases on the function of the GH receptor remain to be determined.

Conclusion. This work demonstrates that GH promotes the phosphorylation of tyrosyl residues in GH receptors in fibroblast and adipocyte forms of 3T3-F442A cells. It will be interesting to determine whether GH-promoted tyrosyl phosphorylation is part of the pathway for expression of the effects of GH on differentiation and cellular metabolism in these cells.

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Registry No. GH, 9002-72-6; Tyr, 60-18-4.

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