Growth Hormone-promoted Tyrosyl Phosphorylation of a 121-kDa Growth Hormone Receptor-associated Protein*

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Previous work in multiple cell types has shown that endogenous GH receptors, as well as the cloned liver GH receptor, associate with a tyrosine kinase. However, in SDS-PAGE gels of highly purified, kinase-active GH receptor preparations from 35S-labeled 3T3-F442A cells, only one broad band was detected corresponding to the molecular weight of the GH receptor rather than two bands which might be expected to result from a kinase-receptor heterocomplex. In the present study, a transfected Chinese hamster ovary (CHO) cell line (CHO4) that expresses an 84-kDa GH receptor rather than a 121-kDa GH receptor was used to examine whether the GH receptor might form a complex with a protein (e.g. tyrosine kinase) that co-migrates on SDS-polyacrylamide gel electrophoresis gels with the endogenous GH receptor (M, 121,000) in 3T3-F442A cells. GH-GH receptor complexes were immunoprecipitated with anti-GH antibody from GH-treated CHO4 cells and incubated with [γ-32P]ATP. 32P was incorporated into a 121-kDa protein as well as the 84-kDa GH receptor. Phosphorylation of both the 84-kDa GH receptor and the 121-kDa protein was on tyrosyl residues as determined by Western blotting with anti-phosphotyrosine antibody. The 121-kDa protein does not appear to bind GH. It was also not detected in the immunoprecipitate when cells had not been incubated with GH or when untransfected CHO cells were used. These findings suggest that in CHO4 cells, the 121-kDa protein is precipitated by the GH antibody because of its ability to form a complex with the GH receptor (p84). Western blot analysis of whole cell lysates using anti-phosphotyrosine antibody revealed that GH promotes the tyrosyl phosphorylation of a 121-kDa protein and several other proteins (p97, p42, p39) in a dose- and time-dependent fashion. Taken together, these findings are consistent with either p121 being the tyrosine kinase that complexes with the GH receptor and is activated in response to GH binding or with p121 forming a ternary complex with both the GH receptor and a tyrosine kinase and serving as a substrate of the GH receptor-associated tyrosine kinase.

Although GH has been known for many years to be a primary determinant of body growth and a modulator of overall lipid, protein, and carbohydrate metabolism (1–4), little is known about the cellular mechanisms responsible for these diverse responses. The GH receptor cloned from liver shows no sequence homology to receptors with known signal transduction pathways (5). However, results from previous studies from our laboratory indicated that in multiple cell types, endogenous GH receptor as well as the cloned liver GH receptor copurifies with tyrosine kinase activity and is phosphorylated on tyrosyl residues (6–9). In cell lines transfected with the cDNA for the liver GH receptor, phosphorylation of the receptor did not correlate with the amount of GH receptor expressed. Instead, phosphorylation was found to vary substantially with the cell type (9). Furthermore, in one cell type tested (3T3-F442A cells), tyrosine kinase activity associated with the GH receptor was increased in response to GH binding (10). Taken together, these findings support the hypothesis that the GH receptor forms a complex with a tyrosine kinase and raise the possibility that activation of a GH receptor-associated tyrosine kinase is an early or perhaps initiating step in GH signal transduction. If the GH receptor forms a complex with a tyrosine kinase, then one would expect to see two proteins (i.e. the GH receptor and the tyrosine kinase) in SDS-PAGE gels of kinase-active GH receptor preparations. However, only one rather broad band was consistently visible on SDS-PAGE gels of highly purified (estimated 30,000–40,000-fold) GH receptor isolated from 35S-labeled 3T3-F442A cells (7, 10). Proteins in this band, which migrate with a size appropriate for the endogenous GH receptor in these cells (M, 121,000), incorporated 32P into tyrosyl residues when the purified GH receptor preparation was incubated with [γ-32P]ATP. Since the GH receptor and tyrosine kinase appear to be separate proteins, these findings suggest that the GH receptor-associated kinase: 1) has the same apparent molecular weight as the GH receptor and thus cannot be distinguished from the GH receptor by one-dimensional SDS-PAGE; 2) has a low rate of turnover or contains few or no methionine or cysteine residues and has therefore incorporated little 35S during the overnight incubation; or 3) is present in the highly purified GH-receptor preparations at such low levels as to be undetectable, indicating a kinase:receptor stoichiometry substantially less than 1:1. To determine if the GH receptor forms a complex with a nonreceptor protein (e.g. kinase) of M, ~120,000, we have taken advantage of a CHO cell line (CHO4) which was stably transfected with a cDNA containing the entire coding region

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† The abbreviations used are: GH, growth hormone; hGH, human growth hormone; DTT, dithiothreitol; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IL, interleukin; CHO, Chinese hamster ovary; GM-CSF, granulocyte-macrophage colony-stimulating factor.
of the cloned rat liver GH receptor but which expresses an 84-kDa GH receptor (p84) (11). This receptor is smaller than both endogenous and transfected rodent GH receptors (m, 113,000–130,000) expressed in other cell lines (8, 9). The molecular basis for the smaller size of the 84-kDa GH receptor in CHO4 cells is not known since other CHO clones transfected simultaneously with the same GH receptor cDNA express the larger 121-kDa GH receptor and the size of the GH receptor mRNA expressed in the CHO4 cells is the same as that expressed by the CHO cells expressing the larger 121-kDa receptor (Ref. 9, data not shown). Despite its smaller size, this GH receptor is a functional receptor, since it binds GH and elicits effects of GH on protein synthesis, lipid synthesis, lipolysis, and MAP kinase activity (11, 12). We show that when GH–GH receptor complexes are immunoprecipitated with anti-GH antibody from GH-treated CHO4 cells, the 84-kDa GH receptor copurifies with a 121-kDa protein (p121). In an in vitro kinase assay, both the GH receptor and the 121-kDa protein are phosphorylated on tyrosyl residues. Tyrosyl phosphorylation of a 121-kDa protein in intact cells is also GH-dependent. We speculate that the 121-kDa GH receptor-associated protein is the GH receptor-associated tyrosine kinase, although we cannot rule out the possibility that p121 is a substrate of the kinase that forms a ternary complex with the GH receptor and a GH receptor-associated tyrosine kinase.

EXPERIMENTAL PROCEDURES

Materials—The stock of 3T3-F442A fibroblasts was kindly provided by Dıs. R. Corin and M. Sonenberg (Memorial Sloan-Kettering Cancer Center, New York, NY) who obtained them from Dr. H. Green (Harvard University, Cambridge, MA). Recombinant DNA plasmid 22,000-Dalton GH was a gift of Mr. R. Lilly Co., and rabbit anti-GH antibody (NIDDK anti-GH IC-3, lot C118981A) came from the National Institute of Diabetes and Digestive and Kidney Diseases/National Hormone and Pituitary Program, University of Maryland School of Medicine (Baltimore, MD). Rabbit anti-phosphotyrosine antibody (aPY-Shafer) was kindly supplied by Dr. J. A. Shafer (Merck, Sharpe and Dohme Research Laboratory, West Point, Pa.), and monoclonal anti-phosphotyrosine antibody (aPY-4G10) was purchased from UBI. Rabbit anti-GH receptor antiserum (GHBP-poly) and monoclonal anti-phosphotyrosine antibody (aPY-4G10) was pur- chase from UBI. Rabbit anti-GH receptor antiserum (GHBP-poly) was a gift of Dr. W. R. Baumbach (American Cyanamid, Princeton, NJ). Recombinant protein A-agarose was from Repligen, Triton X-100 (Surfact-Amps X-100) and DSS from Pierce Chemical Co., and leupeptin were purchased from Boehringer Mannheim. Molecular weight standards and protein assay reagents from Bio-Rad. Aprotinin and DTT, 0.1% Triton X-100, pH 7.6). In vitro phosphorylation was carried out by incubating the immunomasms at 30 °C with [32P] ATP (~200 cpm, 4 μM) in buffer C with 250 μg/ml leupeptin and 250 ng/ml aprotinin as described previously (7). After 10 min, the reaction was quenched by the addition of 12 ml of ice-cold NHT buffer containing 10 mM EDTA, pH 7.6, followed by extensive washing with NHT buffer containing 0.5 mM DTT. Immunoprecipitated proteins were eluted from the immunomasms by boiling in 200 μl of SDS-PAGE sample buffer (150 mM Tris, pH 6.8, 10% glycerol, 5% SDS, 0.03 mg/ml bromphenol blue, and 360 mM β-mercaptoethanol), and analyzed by SDS-PAGE (see below).

RESULTS

Preparation of Partially Purified GH Receptor—Confluent CHO cells and 3T3-F442A fibroblasts were incubated in serum-free medium overnight (16–20 h). As described previously (7), cellular proteins were solubilized in ice-cold HVTDP buffer (25 mM HEPES, 2 mM Na2VO4, 0.1% Triton X-100, 0.5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at 200,000 × g at 4 °C for 1 h. The supernatant was incubated with anti-GH antiserum (1:5,000 or 1:10,000) for 2 h at 4 °C in an end-over-end mixer. Immune complexes were precipitated with immobilized protein A. The immunomasms were extensively washed with NHT buffer (150 mM NaCl, 50 mM HEPES, 0.1% Triton X-100, pH 7.6) plus 0.5 mM DTT.

In Vitro Kinase Assay—Immunomasms were washed twice with buffer C (50 mM HEPES, 100 mM NaCl, 6.25 mM MnCl2, 0.5 mM DTT, 0.1% Triton X-100, pH 7.6). In vitro phosphorylation was carried out by incubating the immunomasms at 30 °C with [32P] ATP (~200 cpm, 4 μM) in buffer C with 250 μg/ml leupeptin and 250 ng/ml aprotinin as described previously (7). After 10 min, the reaction was quenched by the addition of 12 ml of ice-cold NHT buffer containing 10 mM EDTA, pH 7.6, followed by extensive washing with NHT buffer containing 0.5 mM DTT. Immunoprecipitated proteins were eluted from the immunomasms by boiling in 200 μl of SDS-PAGE sample buffer (150 mM Tris, pH 6.8, 10% glycerol, 5% SDS, 0.03 mg/ml bromphenol blue, and 360 mM β-mercaptoethanol), and analyzed by SDS-PAGE (see below).

Formation of Cross-linked 121-kDA GH Receptor Complexes—Human GH was labeled with 125I by Dr. J. Smart (University of Michigan) to an estimated specific activity of 250–300 cpm/ng using a modification of the chloramine-T procedure (13). 125I-GH was cross-linked to GH receptors as described previously (6). Briefly, cells were deprived of serum overnight as described above. 125I-GH (5 × 105 cpm/100-mm dish, ~6 ng/ml) in Krebs-Ringer phosphate buffer, 1% bovine serum albumin was added to the cells in the presence or absence of 4 μg/ml unlabelled hGH and incubated for 1 h at 25 °C. After extensive washing, DSS (0.4 mM final concentration) was added for 15 min at 8 °C to cells in Krebs-Ringer phosphate buffer. Cells were solubilized using HVTDP, and cellular proteins were analyzed by SDS-PAGE.

Preparation of Partially Purified GH Receptor—CHO cells were lysed with boiling SDS lysis buffer (63 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 25% (v/v) glycerol, 10 mg/ml bromphenol blue, 100 mM DTT, 5% (v/v) β-mercaptoethanol, 1 mM Na2VO4, 0.1 mM Na3VO4, 100 μg/ml leupeptin, and 100 μg/ml aprotinin) as described previously (15). Whole cell lysates were subjected to SDS-PAGE, and proteins were transferred electrophoretically at 4 °C to PVDF paper for 1 h at 1 amp or 100 V. Membranes were washed twice with NHT buffer (150 mM NaCl, 50 mM HEPES, 0.1% Triton X-100) for 1 h at room temperature with TBS (10 mM Tris-HCl, 135 mM NaCl, pH 7.4) containing 0.1% Tween-20 and 4% chicken egg ovalbumin before an overnight incubation with aPY-4G10 (200 mg/ml) or aPY-Shafer (840 mg/ml). After extensive washing with TBS-0.1% Tween-20 followed by washing with TBS, membranes were incubated with anti-rabbit IgG conjugated to horseradish peroxidase (for aPY-Shafer) or anti-rabbit IgG conjugated to horseradish peroxidase (1:5000) for 2 h. Proteins were visualized using the enhanced chemiluminescence detection system. Alternatively, partially purified GH receptor preparations were subjected to SDS-PAGE, transferred to PVDF paper, and processed as above.

Cell Culture—CHO cells were cotransfected with plasmid pML108 and pBlBP-1 as described previously (11). Plasmid pML108 contains the cDNA for the entire coding region of the rat liver GH receptor driven by the SV40 enhancer and the Zn+2-inducible human metallothionein IIa promoter. Plasmid pBlBP-1 contains a thyminase kinase promoter fused to the bacterial neocynin phosphotransferase gene conferring G418 resistance. Two stably transfected cell lines were used: 1) CHO4 cells expressing an 84-kDa GH receptor (11); and 2) CHO cells expressing a 121-kDa GH receptor (9). CHO cells were cultured at 37 °C under 5% CO2-95% air in Ham’s F-12 medium containing 1.8 g/liter glucose and 10% fetal calf serum. Medium was supplemented with 1 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml amphotericin B. G418 (500 μg/ml) was added to the medium of transfected CHO cells used for passaging. Experimental plates and untransfected cells were maintained in medium without G418. 3T3-F442A cells were grown at 37 °C under 10% CO2-90% air in Dulbecco’s modified Eagle’s medium containing 2 g/liter glucose and 10% calf serum plus l-glutamine and antibiotics as described previously (6).

2 C. Möller, M. Emtner, P. Arner, and G. Norstedt, manuscript in preparation.
pg/ml unlabeled hGH and then DSS (0.4 mM) was added for 15 min under conditions in which proteolysis is likely to occur partially proteolyzed '125I-hGH-receptor complexes, since it was present in all cell types in this particular experiment. This includes 3T3-F442A cells in which 125I-labeled proteins of this size only appear under conditions in which proteolysis is likely to occur (e.g. experiments in which the sample processing time is extended due to a large number of samples) (compare to Fig. 1 of Ref. 6).

Based upon these findings in 3T3-F442A and CHO cells, the 84-kDa GH receptor (p84) was expected to be the only protein phosphorylated in the in vitro kinase assay when CHO cells were used as starting material. However, when CHO cells were incubated with GH and the GH receptor was immunoprecipitated on immobilized anti-GH antibody and incubated with [γ-32P]ATP, two phosphoproteins (p121 and p84) were detected (Fig. 2, lane 5). Neither phosphoprotein was detected when cells had not been incubated with GH (Fig. 2, lane 6), consistent with both proteins being precipitated specifically by the anti-GH antibody. The identity of the lower band (p84) as the expressed cloned liver GH receptor is based on the observation that cross-linked 125I-hGH-receptor complexes in CHO cells migrate with M, 101,000 (Fig. 1, lane 3) which suggests that the molecular weight of the GH receptor is ~80,000. Furthermore, anti-GH receptor antibody recognizes an 84-kDa protein in Western blots (data not shown). The other band, pp121, is unlikely to be a GH receptor. Based on the cross-linking studies in Fig. 1 (lane 3) and Western blots using anti-GH receptor antibody, pp121 is too big to be the expressed liver GH receptor in these cells. It is also unlikely to be an endogenous GH receptor, because CHO cells do not express endogenous GH receptors. No GH binding is detectable in untransfected CHO cells (Ref. 11 and data not shown), nor are any 134-kDa cross-linked 125I-hGH-receptor complexes observed when untransfected CHO cells are incubated with 125I-hGH and cross-linking reagent (Fig. 1, lane 1). Although a small amount of 125I migrating with M, ~130,000 was observed for CHO cells in some cross-linking experiments (Fig. 1, lane 3), the finding that p121 is specifically immunoprecipitated by GH antibody and prominently phosphorylated in the presence of [γ-32P]ATP (Fig. 2, lane 5) but at best only inefficiently cross-linked to 125I-hGH-receptor complexes in CHO cells (Fig. 1, lane 3) is most consistent with p121 being a non-GH binding protein that complexes with the GH receptor.

Both p121 and the GH Receptor (p84) Isolated from CHO Cells Are Phosphorylated on Tyrosyl Residues in an in Vitro Kinase Assay—Western blot analysis using anti-phosphotyrosine antibody was used to determine whether phosphorylation of p121 and p84 during the in vitro kinase assay is on tyrosyl residues and whether these proteins are phosphorylated on tyrosyl residues in the intact cell. CHO cells were incubated with 100 ng/ml GH and GH-GH receptor complexes were immunoprecipitated using anti-GH antibody. Half of the sample was analyzed directly by Western blot analysis using an anti-phosphotyrosine antibody to evaluate whether p84 and p121 are phosphorylated in vivo on tyrosyl residues. The other half of the sample was incubated with unlabeled ATP (4 μM) for 10 min at 30°C as described for the in vitro kinase assay and then analyzed by Western blot assay using anti-phosphotyrosine antibody to determine whether the phosphorylation of p84 and p121 observed in the in vitro kinase assay is on tyrosyl residues. GH-receptor complexes from 3T3-F442A and CHO cells were analyzed in a similar fashion. Fig. 3 (lanes 2, 5, and 8) shows that p121 from CHO4, CHOa, and 3T3-F442A cells incubated with GH
is phosphorylated on tyrosyl residues in vivo. Fig. 3 (lanes 3, 6, and 9) also reveals that in vitro incubation of GH receptor preparation with ATP results in increased tyrosyl phosphorylation of pp121 in all three cell types and the emergence of phosphorylated tyrosyl residues in p84 in the CHO4 cells treated with GH, indicating that both p121 and p84 are phosphorylated on tyrosyl residues in the in vitro kinase assay. Tyrosyl phosphorylation resulting from the in vitro kinase reaction decreased migration of a portion of p121, consistent with the in vitro kinase reaction phosphorylating additional tyrosyl residues in proteins in which at least 1 tyrosyl residue had already been phosphorylated in vivo.

Interestingly, in vivo tyrosyl phosphorylation of p84 from CHO4 cells was not detectable in Fig. 3, lane 5. However, in some experiments in which GH-receptor preparations were analyzed by Western blotting using aPY-4G10, a faint band of M, 84,000 was detectable in overexposed autoradiograms, indicating that the 84-kDa GH receptor from GH-treated cells is most likely also phosphorylated on tyrosyl residues (data not shown). To determine whether the difficulty in detecting the presence of phosphorylated tyrosyl residues in p84 may reside at least in part from poor binding of p84 to PVDF membranes used in the Western blot procedure, p121 and p84 were labeled in vitro with 32P as described for Fig. 2, and subjected to SDS-PAGE, and either analyzed directly by autoradiography or transferred to PVDF paper and washed as for the Western blot assay before autoradiography. A comparison of the relative intensities of 32P-labeled p121 and p84 in both autoradiograms indicated that 32P-labeled 84-kDa GH receptor binds poorly to PVDF paper when compared to 32P-labeled 121-kDa protein (data not shown). The tyrosyl-phosphorylated 84-kDa GH receptor also bound poorly to nitrocellulose (data not shown). Poor binding of the GH receptor to PVDF may explain in part why the amount of tyrosyl-phosphorylated p121 present in the immunoprecipitate from CHO4 cells appears to be as great as or greater than that in CHO1 cells in Fig. 3, even though p121 in the CHO1 cells consists of two proteins (GH receptor and p121 GHR-associated protein), whereas p121 in the CHO4 cells consists only of p121 GHR-associated protein. However, it should also be noted that: (a) despite the same number of confluent plates being used for both CHO1 and CHO4 cells, the samples are not directly comparable, because the samples were not normalized for cell or GH receptor number; and (b) the CHO4 cells express more GH receptors than the CHO1 cells (see Fig. 1) so that if the cell number were the same in the two samples and if the stoichiometry of GH receptor:p121 GHR-associated protein approaches 1:1, then one would expect to see more tyrosyl-phosphorylated p121 GHR-associated protein in the CHO4 cells than in the CHO1 cells.

A 121-kDa Protein Is Tyrosyl Phosphorylated in Response to GH—To examine whether p121 is tyrosyl-phosphorylated in response to GH, CHO4 cells were incubated at 25 °C with various concentrations of GH (0, 1, 10, 100, and 1000 ng/ml) for 15 min (Fig. 4) or with 100 ng/ml GH for various lengths of time (0, 5, 15, and 60 min) (Fig. 5). Cells were lysed with

![Fig. 3. p121 and GH receptor (p84) from CHO4 cells are phosphorylated in vitro on tyrosyl residues.](image-url)

![Fig. 4. Dose response for GH-promoted tyrosyl phosphorylation of p121 and other cellular proteins in CHO cells expressing the cloned liver GH receptor.](image-url)

![Fig. 5. Time course of GH-promoted tyrosyl phosphorylation of cellular proteins in CHO cells expressing the cloned liver GH receptor.](image-url)
boiling SDS lysis buffer, and aliquots of the whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using anti-phosphotyrosine antibody (α-PY-4G10 (Figs. 4 and 5A) or α-PY-Shafer (Fig. 5B)) to visualize tyrosyl-phosphorylated proteins. Figs. 4 and 5 show that GH stimulates tyrosyl phosphorylation of a 121-kDa protein in CHO4 cells in a dose-dependent (Fig. 4) and time-dependent (Fig. 5) fashion. This 121-kDa protein comigrates with the 121-kDa protein present in anti-GH receptor immunoprecipitates, consistent with pp121 in the Western blots and pp121 in the anti-GH antibody immunoprecipitates being the same protein. Tyrosyl phosphorylation of pp121 is detectable after 15 min with 10 ng/ml GH and is maximal with 100 ng/ml GH (Fig. 4, lanes 6–10). When cells are incubated with 100 ng/ml hGH, the stimulatory effect is apparent at the earliest time tested (5 min) (Fig. 5, lane 6). Increased tyrosyl phosphorylation of pp121 in response to GH was also observed in CHO4 cells expressing the larger sized (121 kDa) GH receptor (Fig. 5B, lanes 9–12) although these cells show the relative contributions to that increase of the 121-kDa GH receptor and the nonreceptor 121-kDa protein cannot be determined. GH did not stimulate the tyrosyl phosphorylation of a 121-kDa protein in untransfected CHO cells (Fig. 4, lanes 1–5 and Fig. 5B, lanes 1–4) as predicted from the absence of GH receptors in these cells.

When CH04 and CHO4 cells were incubated with GH, three proteins (p97, p42, and p39) in addition to p121 showed increased tyrosyl phosphorylation in response to GH (Figs. 4 and 5A). The 42- and 39-kDa proteins are thought to be ERK5 1 and 2 based upon their comigration with proteins identified by anti-MAP kinase antibodies in CHO4 and 3T3-F442A cells. GH-dependent tyrosyl phosphorylation of an 84-kDa protein is not detectable, presumably due at least in part to the poor ability of the GH receptor to bind to PVDF paper (see above).

**DISCUSSION**

*Identification of a 121-kDa Protein That Forms a Complex with GH Receptor*—In the present study, we demonstrate that anti-GH antibody specifically precipitates two proteins (p84 and p121) from CHO4 cells incubated with GH. Both proteins are phosphorylated on tyrosyl residues when incubated *in vitro* with [γ-32P]ATP. We also demonstrate that the 121-kDa protein, as well as several other proteins, are tyrosyl-phosphorylated in response to GH. The 84-kDa protein is believed to be the GH receptor expressed in CHO4 cells based on the findings that: 1) cross-linked 125I-hGH-receptor complexes in these cells migrate with a Mr (101,000), consistent with a GH receptor of Mr ~80,000; and 2) p84 comigrates with a protein that binds in Western blot assays to an anti-αGH receptor antibody. The findings that neither GH binding nor 134-kDa cross-linked 125I-hGH-receptor complexes are detectable in untransfected CHO cells and that the anti-GH receptor antibody does not detect a 121-kDa protein in Western blots of CHO4 cells suggest that p121 in the CHO4 cells is a nonreceptor protein. Interestingly, a faint band of ~130,000 labeled by 125I was sometimes detected in cross-linking studies of CHO4 cells. We feel it unlikely that this faint band represents endogenous 121-kDa GH receptor cross-linked to 125I-hGH since it was not present in untransfected CHO cells. One could hypothesize that GH binding to the cloned liver GH receptor expressed in CHO4 cells somehow induces the expression of the endogenous 121-kDa GH receptor. However, in mouse L cells in which the endogenous GH receptor mRNA can be distinguished from expressed cloned liver GH receptor mRNA by size, expression of the cloned mouse liver GH receptor decreased, rather than increased, levels of endogenous GH receptor mRNA (data not shown). Similarly, expression of a truncated rat liver GH receptor in RIN5-AH cells decreased rather than increased the number of endogenous GH receptors as assessed by cross-linking 125I-hGH to GH receptors in intact cells (17). A more likely explanation for the presence of a faint band migrating with Mr, 134,000 in CHO4 cells is that some 125I-hGH became directly cross-linked to p121 due to its proximate location in the GH receptor/p121 complex. Precedence for this is provided by the observation that in cross-linking studies, a small amount of 125I-insulin can be cross-linked to the non-insulin binding subunit of the insulin receptor (18). In light of this evidence favoring p121 being a non-GH binding protein, the finding that p121 is immunoprecipitated by anti-GH antibody from lysates of GH-treated cells is most consistent with it forming a complex with the GH receptor (p84).

**Binds p121 GH-Receptor-associated Protein in CHO4 Cells**—Phosphorylated on Tyrosyl Residues—The present study provides strong evidence that p121 GH-receptor-associated protein is tyrosyl-phosphorylated in response to GH and is phosphorylated in the *in vitro* kinase assay by the GH receptor-associated tyrosine kinase. It also provides strong evidence that, at least in CHO4 cells, the GH receptor is phosphorylated by the GH receptor-associated tyrosine kinase in the *in vitro* kinase assay. Evidence also supports the tyrosyl phosphorylation of the GH receptor from 3T3-F442A cells by the GH receptor-associated tyrosine kinase in the *in vitro* kinase assay. In experiments not shown, GH-receptor complexes were immunoprecipitated using anti-GH antibody, incubated with [γ-32P]ATP, boiled for 2 min in 1% SDS, 10 mM EDTA to dissociate protein complexes, and then incubated with anti-GH receptor antibody. The ability of immobilized anti-GH receptor antibody to precipitate a substantial portion of the 32P-labeled p121 provides evidence that at least a portion of the 32P was incorporated into the GH receptor rather than into p121 GHR-associated protein. In contrast to these results using the *in vitro* kinase assay, the Western blot results shown in Figs. 3–5 would seem to suggest that, when GH is added to the intact cell, very few if any GH receptors become phosphorylated on tyrosyl residues since very little 84-kDa protein is detected by the α-PY antibody in the Western blot assays. However, the Western blot results are misleading because poor binding of the GH receptor to PVDF membranes results in an artefactually low signal. The finding that even with poor binding of the GH receptor to PVDF membranes, a faint signal corresponding to the 84-kDa GH receptor (discussed under "Results") or a more substantial signal corresponding to an 80-kDa truncated GH receptor missing approximately half its cytoplasmic domain is detectable in some Western blots using α-PY antibody indicates the presence of tyrosyl residues in these GH receptors. Experiments using cross-linked 125I-hGH-GH receptor complexes also indicate the presence of phosphorylated tyrosyl residues in the GH receptor in 3T3-F442A cells. In previous studies showing specific binding (3–20%) of 125I-hGH-GH receptor complexes to immobilized anti-phosphotyrosine antibodies (6, 9), solubilization conditions were sufficiently mild (0.1 or 1% Triton X-100) that one could argue that the complexes bound to the antibody indirectly through their tight association with the

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1. J. R. Stubbart, W. Smith, F. Talamantes, and C. Carter-Su, unpublished observation.

2. X. Wang, D. Meyer, G. Campbell, J. Schwartz, N. Billestrup, G. Norstedt, and C. Carter-Su, manuscript in preparation.
tyrosyl-phosphorylated p121 GHR-associated protein. To counteract this argument, we solubilized cross-linked (with disuccinimidyl suberate) \(^{125}\)I-hGH-receptor complexes from intact cells with 0.1% Triton X-100 and then boiled the cell lysate in a buffer containing 0.1% Triton X-100, 0.5% SDS, and 10% \(\beta\)-mercaptoethanol to dissociate protein complexes. The sample was diluted 1:26 with buffer containing 0.1% Triton X-100 and then incubated with immobilized \(\alpha\)PY-Shafer antibody. Even under these harsh solubilization conditions, some \((\sim 1\%) \)\(^{125}\)I-hGH-receptor complexes were recognized by the \(\alpha\)PY-Shafer antibody. Taken together, these results provide evidence that at least a portion of the GH receptors are tyrosyl-phosphorylated in GH-treated cells. However, they do not provide insight into the relative proportions of phosphorylated tyrosyl residues present in the GH receptor and in p121 GHR-associated protein. This delineation will require development of techniques that separate the two proteins without affecting their relative recoveries or phosphorylation states. Determination of whether tyrosyl phosphorylation of the GH receptor is important for eliciting responses to GH awaits an examination of whether receptors lacking specific tyrosyl residues are able to function normally. p121 GHR-associated Protein Is the GH Receptor-associated Tyrosine Kinase or a Substrate of the GH Receptor-associated Tyrosine Kinase That Forms a Ternary Complex with the Kinase and the GH Receptor—While the current study revealed the presence of a 121-kDa GHR-associated protein which can be tyrosyl-phosphorylated in \(\textit{in vitro}\) and \(\textit{in vivo}\), whether p121 is the GH receptor-associated kinase is not yet clear. Consistent with p121 being the GH receptor-associated tyrosine kinase, several lines of evidence indicate that the tyrosine kinase responsible for GH receptor phosphorylation in CHO4 and 3T3-F442A cells is present in a complex with GH receptor and p121 GHR-associated protein. First, in CHO4 cells the GH receptor-p121 complex purified by immunoadsorption to anti-GH antibody catalyzes the \(\textit{in vitro}\) phosphorylation of tyrosyl residues in both proteins in the complex. Second, dilution by 100-fold of GH-receptor preparations from GH-treated 3T3-F442A cells does not decrease the rate of GH receptor/p121 phosphorylation in an \(\textit{in vitro}\) kinase assay (10). Finally, the amount of tyrosine kinase activity present in anti-GH receptor immunoprecipitates from 3T3-F442A cells, assessed by the amount of \(^{32}\)P transferred from \([\gamma-\text{P}]\text{ATP}\) to a synthetic substrate or to GH receptor/ p121, is greater when cells are incubated with GH (10). Since previous studies indicate that the GH receptor does not possess intrinsic tyrosine kinase activity (9), it is appealing to hypothesize that p121 is the GH receptor-associated tyrosine kinase which phosphorylates the GH receptor. A tyrosine kinase of \(M\), 121,000 would explain why two proteins, one corresponding to the GH receptor and the other to the tyrosine kinase responsible for GH receptor phosphorylation, could not be distinguished in SDS-PAGE gels of highly purified, kinase-active, GH receptor preparations from \(^{35}\)S-labeled 3T3-F442A cells; cells in which the GH receptor itself migrates with \(M\), 121,000. The following findings using 3T3-F442A cells (19) are consistent with p121 being a tyrosine kinase with a central role in GH signaling: 1) in time course studies, tyrosyl phosphorylation of 121-kDa protein is detected in response to GH as early as or earlier than tyrosyl phosphorylation of other proteins; 2) increased tyrosyl phosphorylation of the 121-kDa protein is detected at lower concentrations of GH (0.5 ng/ml) than required for detection of other phosphoproteins; 3) p121 is one of the few proteins phosphorylated on tyrosyl residues in response to GH that is not phosphorylated on tyrosyl residues in response to other growth factors whose receptors have intrinsic tyrosine kinase activity (e.g., receptors for epidermal growth factor, platelet-derived growth factor, insulin-like growth factor-1); and 4) all three of the tyrosine kinase inhibitors identified as inhibitors of the GH receptor-associated tyrosine kinase using the \(\textit{in vitro}\) kinase assay prevent GH-promoted tyrosyl phosphorylation of p121 when added to cells. In contrast, other inhibitors of tyrosine kinases that are ineffective against the GH receptor-associated kinase do not block tyrosyl phosphorylation of p121 in response to GH.

The cloned liver GH receptor is a member of the cytokine/hematopoietic receptor superfamily, with the prolactin receptor being the member most closely related to the GH receptor. This receptor family, which includes receptors for ILS 2–7, erythropoietin, granulocyte colony stimulating factor, GM-CSF, and ciliary neurotrophic factor (20–30) is defined by homologies in the extracellular domain. However, receptors in this family may also share some common characteristics in their intracellular signaling. In fact, binding of ligand to a number of these receptors stimulates tyrosine phosphorylation of cellular proteins (e.g. receptors for GH, prolactin, ILS 2–4, and 7, GM-CSF, erythropoietin (16, 19, 31–35)). In a manner similar to that shown for the GH receptor (7), the receptors for IL-2 and erythropoietin have been shown to associate with a tyrosine kinase (36, 37). The tyrosine kinase has been tentatively identified (as Lck, a 96-kDa member of the Src family) for only one member of this receptor family, IL-2 receptor (38, 39). Whether a member of the Src family of kinases associates with the GH receptor is not known. However, no phosphoproteins migrating with a size appropriate for members of this family have been observed in kinase-active GH receptor preparations (6–9).

Proteins similar in size \((M, 130,000–140,000)\) to the 121-kDa protein identified in this study have been identified in association with the receptors for other members of the cytokine/hematopoietic receptor family, including the receptors for IL-3, IL-5, IL-6, GM-CSF, and erythropoietin (37, 40–42). These proteins which associate with the receptor as a complex are thought to play a role in ligand binding and/or signal transduction. While the 130-kDa proteins that associate with the receptors for IL-3, IL-5, IL-6, and GM-CSF have been cloned and are assumed not to be tyrosine kinases based upon sequence analysis (40–42), it is not known whether they are phosphorylated on tyrosyl residues in response to ligand binding. In contrast, erythropoietin receptor-associated p130 is tyrosyl-phosphorylated in response to erythropoietin (57) and is a likely candidate for the erythropoietin receptor-associated tyrosine kinase. It is therefore intriguing to speculate that p121 identified in this study as a GH receptor-associated protein is a member of a family of proteins that associate with members of the cytokine/hematopoietic receptor family and are important for signal transduction, either as tyrosine kinases or as signal transducing proteins that are activated upon phosphorylation by the appropriate ligand-activated tyrosine kinase.

While we favor the interpretation that p121 is the GH receptor-associated tyrosine kinase, our data cannot rule out the alternative hypothesis that p121 forms a ternary complex with the GH receptor and the tyrosine kinase, and serves as a substrate of the GH receptor-associated tyrosine kinase. This is a less appealing hypothesis, since, if p121 is not the kinase, the inability to detect more than one band when highly purified kinase-active GH receptor is purified from \(^{35}\)S-labeled 3T3-F442A cells and subjected to SDS-PAGE and autoradiography (7, 10) would imply that the GH receptor-associated tyrosine kinase has an extremely low rate of turnover; has
few or no cysteine or methionine residues; or that the ratio of kinase to GH receptor/p121 complex in the highly purified kinase-active GH preparations is so low that the kinase is not detectable.

**GH Signaling through GH Receptor-associated Tyrosine Kinase**—In addition to p121, we show here at least three other cellular proteins (p97, p42, and p39) that become tyrosyl-phosphorylated in response to GH in CHO and CH04 cells expressing the cloned liver GH receptor. Two of these proteins (p42 and p39) comigrate with proteins identified in 3T3-F442A cells as ERK1 and ERK2 (16, 15). This confirms the finding of Möller and colleagues (12) that GH stimulates tyrosyl phosphorylation of MAP kinase in CH04 cells expressed in response to GH receptor/L. Mathews for helpful discussions; Drs. G. Campbell and L. Argetsinger, L. S., and Shafer, J. A. (1989) J. Biol. Chem. 264, 18564–18661

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