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Growth Hormone, Interferon-γ, and Leukemia Inhibitory Factor Promoted Tyrosyl Phosphorylation of Insulin Receptor Substrate-1*

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The identification of JAK2 as a growth hormone (GH) receptor-associated, GH-activated tyrosine kinase has established tyrosyl phosphorylation as a signaling mechanism for GH. In the present study, GH is shown to stimulate tyrosyl phosphorylation of insulin receptor substrate 1 (IRS-1), the principle substrate of the insulin receptor. Tyrosyl phosphorylation of IRS-1 is a critical step in insulin signaling and provides binding sites for proteins with the appropriate Src homology 2 domains, including the 85-kDa regulatory subunit of phosphatidylinositol (PI) 3′-kinase. In 3T3-F442A fibroblasts, GH-dependent tyrosyl phosphorylation of IRS-1 was detected by 1 min and at GH concentrations as low as 5 ng/ml (0.23 nM). Tyrosyl phosphorylation of IRS-1 was transient, with maximal stimulation detected at 30 min and diminished signal detected at 60 min. The ability of GH receptor (GHR) to transduce the signal for IRS-1 tyrosyl phosphorylation is mediated by the intracellular region of GHR between amino acids 295 and 380 by a mechanism not involving the two tyrosines in this region. This region of GHR is required for GH-dependent JAK2 association and activation (VanderKuur, J. A., Wang, X., Zhang, L., Campbell, G. S., Allevato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1994) *J. Biol. Chem.* 269, 21709–21717). When other cytokines that activate JAK2 were tested for the ability to stimulate the tyrosyl phosphorylation of IRS-1, stimulation was detected with interferon-γ and leukemia inhibitory factor. The correlation between JAK2 tyrosyl phosphorylation and IRS-1 tyrosyl phosphorylation in response to GH, interferon-γ, and leukemia inhibitory factor and in cells expressing different GHR mutants, provides evidence that IRS-1 may interact with JAK2 or an auxiliary molecule that binds to JAK2. GH is also shown to stimulate binding of IRS-1 to the 85-kDa regulatory subunit of PI 3′-kinase. The ability of GH to stimulate tyrosyl phosphorylation of IRS-1 and its association with PI 3′-kinase provides a biochemical basis for responses shared by insulin and GH including the well characterized insulin-like metabolic effects of GH observed in a variety of cell types.

A major effect of GH[1] in glycemic control is to oppose the action of insulin. GH stimulates hepatic glucose production. It also stimulates lipolysis and decreases glucose utilization in fat tissue. However, GH is also known to have short term effects that mimic insulin actions in tissues that have been deprived of GH (1). This pattern of rapid, transient insulin-like effects and delayed onset prolonged anti-insulin effects is also detected in 3T3-F442A adipocytes. For example, in cells that have been deprived of GH, GH initially stimulates glucose uptake. However, with longer term GH incubation, glucose uptake becomes depressed (2, 3). The existence of the insulin-like effects of GH suggests that GH may utilize some signaling molecules utilized by insulin.

IRS-1 is a cytosolic protein that becomes tyrosyl phosphorylated at multiple sites in response to insulin (4) and insulin-like growth factor-1 (IGF-1) (5). Tyrosyl phosphorylation of IRS-1 mediates biologic responses to insulin and IGF-1, including mitogenic effects (6, 7) and activation of PI 3′-kinase (8, 9), which is thought to be required for translocation of GLUT 4 glucose transporters (10–12). Tyrosyl phosphorylation of IRS-1 provides binding sites for specific proteins containing Src homology-2 domains, including the 85-kDa regulatory subunit of PI 3′-kinase (4, 13), GRB-2 (14, 15), the tyrosine phosphatase SHPTP2 (16, 17), and Nck (18).

Unlike the receptors for insulin (19) and IGF-1 (20, 21), GHR does not contain an intrinsic tyrosine kinase (22). However, ligand binding to GHR activates JAK2 tyrosine kinase (23). In addition to GHR, a number of other receptors in the cytokine/hematopoietin receptor superfamily activate JAK2 in response to ligand binding, including the receptors for erythropoietin, prolactin, interleukin (IL) 3, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, leukemia inhibitory factor (LIF), IL-6, oncostatin M, ciliary neurotrophic factor, and interferon-γ (IFN-γ) (24–29). Thus ligand-dependent tyrosyl phosphorylation of cellular substrates is expected in response to receptor binding by these cytokines. Ligand-dependent tyrosyl phosphorylation of proteins in the

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[1] The abbreviations used are: GH, growth hormone; IGF-1, insulin-like growth factor-1; PI, phosphatidylinositol; GHR, growth hormone receptor; IL, interleukin; LIF, leukemia inhibitory factor; IFN-γ, interferon-γ; CHO, Chinese hamster ovary; hGH, human growth hormone; IRS-1, insulin receptor substrate-1; 4PS, interleukin-4-induced phosphotyrosine substrate.

size range (160–170 kDa) appropriate for IRS-1 has been observed with GH (30) and a number of other ligands that bind to members of the cytokine/hematopoietin receptor family, including LIF, IL-6, ciliary neurotrophic factor, and oncostatin M (31, 32). This suggests that these ligands might utilize IRS-1 as a signaling molecule. In this report, we demonstrate that GH rapidly stimulates both tyrosyl phosphorylation of IRS-1 and the association of PI 3′-kinase with IRS-1. In addition, we determine that the cytoplasmic region of GHR proximal to the membrane (amino acids 295–380) is required for IRS-1 tyrosyl phosphorylation; however, within this region, tyrosines 333 and 338 are not required. The demonstration that LIF and IFN-γ, two cytokines that, like GH, activate JAK2 (29, 26), also stimulate tyrosyl phosphorylation of IRS-1 suggests that signaling by IRS-1 may be common to multiple members of the cytokine/hematopoietin family that activate JAK2.

EXPERIMENTAL PROCEDURES

Materials—Recombinant 22,000-Da hGH and porcine insulin were a gift of the Eli Lilly Co. Human recombinant IGF-1 was from Calbiochem. Recombinant protein A-agarose was from Repligen. Protein assay kit (BCA) was from Pierce. Aprotinin, leupeptin, and Triton X-100 were purchased from Boehringer Mannheim. Bovine serum albumin was from Intergen. Prestained molecular weight standards were from Life Technologies, Inc. Nitrocellulose paper was from Schleicher and Schuell. Chicken egg ovalbumin was purchased from Sigma. The enhanced chemiluminescence (ECL) detection system and x-ray film were from Amersham Corp.

Antisera—Antibody to GH (αGH) (NIDDK-anti-hGH-IC3, lot C11981) came from the National Institute of Diabetes and Digestive and Kidney Diseases/National Hormone and Pituitary Program, National Institutes of Health. Anti-phosphotyrosine antibody (αPY) (4G10) and antiserum to the 85-kDa subunit of PI 3′-kinase ($\alpha p85_{UBI}$), which was used for immunoblotting, were purchased from Upstate Biotechnology, Inc. Affinity-purified antibody prepared against a peptide containing amino acids 146–161 of mouse 85-kDa subunit of PI 3′-kinase (αp85) (9) was used for immunoprecipitation. Protein A-purified polyclonal antibody prepared against recombinant IRS-1 (αIRS-1) (4) was used for immunoprecipitations. Monoclonal antibody raised against recombinant IRS-1 ($\alpha IRS\text{-}1_{1M92-7}$) (8) was used for immunoblotting. Antibody to JAK2 (αJAK2) was prepared in rabbits against a synthetic peptide corresponding to amino acids 758–776 of murine JAK2 (24).

Mutagenesis, Transfection, and Cell Culture—CHO cells expressing full-length rat GHR were developed by co-transfecting CHO cells with plasmids pLM108 and pIBP-1 (33, 34). Plasmid pIBP-1 contains the thymidine kinase promoter fused to the bacterial neomycin phosphotransferase gene conferring G418 resistance. Plasmid pLM108 contains the simian virus 40 enhancer and the Zn^{2+}-inducible human metallothionein IIa promoter driving expression of cDNA coding for full-length rat liver GHR. For CHO cells expressing the various GHR mutants, the GHR cDNA in the pLM108 plasmid was mutated to replace lysine codons 455, 381, 319, or 295 with termination codons, to delete the codons for amino acids 297–311, or to replace tyrosine codons 333 and 338 with codons for phenylalanine, as described previously (34–37). The amino acids in GHR are numbered according to Ref. 38. The binding affinity for hGH was similar for each cell line (33, 34).[2] CHO cells were grown in Ham's F-12 medium containing 1.8 g/liter glucose and 10% fetal calf serum. G418 (500 μg/ml) was added to the medium of transfected CHO cells used for passaging. The stock of 3T3-F442A cells was a kind gift of H. Green (Harvard University, Boston, MA). 3T3-F442A cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 8% calf serum as described previously (39). All cells were cultured at 37 °C under 5% CO_2, 95% air. Media were supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

GH Binding Assay—Human GH labeled with ^{125}I to an estimated specific activity of ~90 μCi/μg using chloramine-T was prepared by the University of Michigan Reproductive Sciences Training Grant Core Facility. GH binding of cell monolayers at 25 °C for a period of 1 h was determined as described previously (35). ^{125}I-GH binding was corrected for nonspecific binding as determined by incubating cell monolayers with ^{125}I-hGH in the presence of 2 μg/ml unlabeled hGH. Binding was normalized to protein content using the Pierce BCA protein assay.

Immunoprecipitation and Western Blotting—Confluent cells were incubated in serum-free medium overnight (16–20 h) as described previously (30). The cells were incubated at 37 °C in a 95% air, 5% CO_2 atmosphere with hGH, cytokine, or growth factor at the concentrations and for the times indicated. The cells were rinsed with three changes of ice-cold PBSV (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4) and scraped in lysis buffer (50 mM Tris, pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) on ice. Cell lysates were centrifuged at 14,000 × *g* for 10 min. The supernatants were incubated on ice for 3 h with the indicated antibody. Immune complexes were collected on protein A-agarose for 1 h at 8 °C, washed 3 times with wash buffer (50 mM Tris, pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4) and boiled for 5 min in a mixture (80:20) of lysis buffer and 250 mM Tris, pH 6.8, 5% SDS, 10% β-mercaptoethanol, 40% glycerol. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis on 3–10% gradient polyacrylamide gels (30:0.5, acrylamide/bisacrylamide). For experiments using CHO cells, the amount of sample added to each lane was normalized to the protein content of the cell supernatant. Proteins were transferred to nitrocellulose at 100 V at 4 °C for 2 h in 25 mM Tris, 190 mM glycine, 20% methanol, 0.02% SDS. Blots were incubated with the appropriate antibody and visualized by ECL detection as described previously (30). To reprobe the blot with a second antibody, the blot was rinsed, incubated in 2% SDS, 60 mM Tris, pH 6.7, 100 mM β-mercaptoethanol at 50 °C for 30 min, reprobed without adding additional primary antibody to check that all antibody had been removed, and then reprobed using the second antibody.

Homology Searches—Pearson and Lipman (40) homology searches of sequences from Genbank (murine GHR (accession number M33324), murine LIF receptor (D26177), murine gp130 (M83336), murine IFN-γ receptor (M25764), murine IFN-γ receptor β-chain (S69336), murine JAK1 (S63728), murine JAK2 (L16956), murine JAK3 (L32955)] were performed with the Wisconsin Package FASTA program (82).

RESULTS

GH Stimulates Tyrosyl Phosphorylation of IRS-1—Signalling utilizing IRS-1 is dependent upon its tyrosyl phosphorylation (4). To determine if IRS-1 is tyrosyl phosphorylated following stimulation of cells with GH, solubilized proteins from 3T3-F442A fibroblasts were immunoprecipitated with αIRS-1 and immunoblotted with αPY. GH-dependent tyrosyl phosphorylation of a protein with a M_r (160,000–170,000) appropriate for IRS-1 is detected by 1 min (the earliest time tested) (Fig. 1, *lane F*) and at physiologically relevant concentrations of GH as low as 5 ng/ml (0.23 nM) (the lowest concentration tested) (Fig. 1, *lanes B–D*). IRS-1 tyrosyl phosphorylation is transient, with maximal stimulation in response to 500 ng/ml GH detected at 30 min. The signal is diminished by 60 min (Fig. 1, *lanes E–J*) with only a minimal further decrease detected at 120 and 240 min (data not shown). The identity of this protein as IRS-1 is further suggested by its comigration with a protein recognized by $\alpha IRS\text{-}1_{1M92-7}$ when the blot was probed with $\alpha IRS\text{-}1_{1M92-7}$ (Fig. 1, *lanes P* and *Q*), with a tyrosyl-phosphorylated protein precipitated by αIRS-1 following stimulation with IGF-1 (Fig. 1, *lane K*), and with insulin (data not shown), known effectors of IRS-1 tyrosyl phosphorylation (5, 4). IGF-1-stimulated tyrosyl phosphorylation of IRS-1 is maximal at 3 min (data not shown). The maximal tyrosyl phosphorylation of IRS-1 in response to GH is 50% of the level observed following treatment with 23 nM IGF-1 for 3 min (Fig. 1) and equivalent to the level observed following 5 min of treatment with 10 or 50 nM insulin (data not shown).

In addition to IRS-1, tyrosyl-phosphorylated proteins with M_r 120,000–130,000 are also detected in αIRS-1 immunoprecipitates from GH-treated cells (Fig. 1, *lanes C, D, G–I*). These proteins comigrate with the tyrosyl-phosphorylated JAK2·GHR complex precipitated using αJAK2 (Fig. 1, *lanes N* and *O*) and αGH (Fig. 1, *lane L*). Similarly, a protein that comigrates with tyrosyl-phosphorylated IRS-1 is detected in αJAK2 immuno-

[2] G. Allevato and N. Billestrup, unpublished observation.

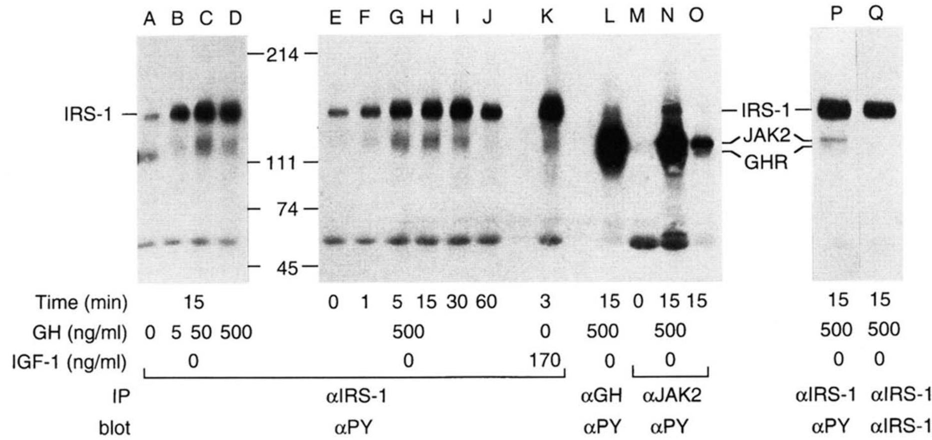

FIG. 1. **GH promoted tyrosyl phosphorylation of IRS-1.** 3T3-F442A fibroblasts were incubated with the indicated concentrations of hGH at 37 °C for 15 min (*lanes A–D*), or with vehicle (*lanes E* and *M*), 500 ng/ml (23 nM) hGH (*lanes F–J, L, N–Q*), or 170 ng/ml (23 nM) IGF-1 (*lane K*) at 37 °C for the times indicated. Whole cell lysates were immunoprecipitated with αIRS-1 (1:250 dilution) (*lanes A–K, P,* and *Q*), αGH (1:8000 dilution) (*lane L*), or αJAK2 (1:250 dilution) (*lanes M–O*). *Lane O* was loaded with one-tenth of the volume of sample loaded in the other lanes. Immunoprecipitated proteins were immunoblotted with αPY (1:7500 dilution) (*lanes A–P*) or αIRS-1_{1M92-7} (1:2000 dilution) (*lane Q*). For *lane P*, the blot was probed first with αIRS-1_{1M92-7}, stripped, and reprobed with αPY. The molecular weight ($\times 10^{-3}$) of protein standards and the migration of IRS-1, JAK2, and GHR are indicated. *Lanes A–D, E–O,* and *P–Q* were from three separate experiments. The band observed at 111 kDa in *lane A* is from the molecular weight standard loaded in the adjacent lane. The faint band detected at 120-kDa in *lane K* was not observed in three other experiments.

precipitates (Fig. 1, *lane N* and see Fig. 5, *lane L*). These results suggest that IRS-1 is present in a complex with JAK2 and GHR; however insufficient protein is co-precipitated to permit identification of these proteins by immunoblotting with the appropriate antibody.

Ability of GH to Induce Association of PI 3′-Kinase with IRS-1—The 85-kDa regulatory subunit of PI 3′-kinase binds to IRS-1 following insulin stimulation (4). To determine if GH promotes the association of the 85-kDa subunit of PI 3′-kinase with IRS-1, IRS-1 was immunoprecipitated from solubilized 3T3-F442A fibroblasts with αIRS-1 and immunoblotted with $\alpha p85_{UBI}$. A GH-dependent increase in the amount of the 85-kDa subunit of PI 3′-kinase, which coprecipitates with IRS-1 was detected (Fig. 2, *lanes A* and *B*). The identity of this protein as the 85-kDa subunit of PI 3′-kinase is suggested by its comigration with a protein immunoprecipitated by αp85 and visualized by $\alpha p85_{UBI}$ in immunoblots (Fig. 2, *lane D*) and the fact that it has a M_r appropriate for the 85-kDa subunit of PI 3′-kinase. The 85-kDa subunit of PI 3′-kinase does not appear to associate with JAK2 as evidenced by the inability to detect p85 following immunoprecipitation with αJAK2 (Fig. 2, *lanes E* and *F*). A GH-dependent increase in PI 3′-kinase associated with IRS-1 demonstrates that not only does GH stimulate tyrosyl phosphorylation of IRS-1, but binding site(s) for PI 3′-kinase are created. Thus, following stimulation with GH, IRS-1 is able to recruit PI 3′-kinase in these cells.

Identification of the Region of GHR Necessary for IRS-1 Tyrosyl Phosphorylation—To begin to understand the molecular basis for the interaction between IRS-1 and GHR, the ability of various mutated GHR to transduce the signal for tyrosyl phosphorylation of IRS-1 was evaluated in CHO cells (mutants summarized in Fig. 3). CHO cells were solubilized and IRS-1 immunoprecipitated using αIRS-1 and then immunoblotted with αPY (Fig. 4*A*). A GH-dependent increase in tyrosyl phosphorylation of IRS-1 is detected in cells expressing GHR_{1-638} (wild-type), GHR_{1-454}, and to a lesser extent GHR_{1-380} (Fig. 4*A*, *lanes A-F*). Binding of ^{125}I-hGH to cells expressing GHR_{1-380} was only 20% of the level detected in cells expressing GHR_{1-638} (Fig. 3). Therefore, the decrease in GH-dependent tyrosyl phosphorylation of IRS-1 detected in cells expressing GHR_{1-380} could at least partially be due to the lower level of GHR expression. No GH-dependent tyrosyl phosphorylation of IRS-1 is

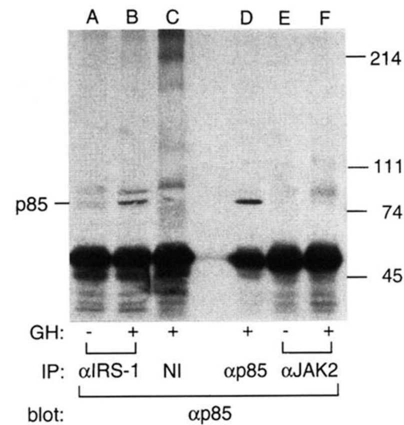

FIG. 2. **GH-dependent association of the 85-kDa regulatory subunit of PI 3′-kinase with IRS-1.** 3T3-F442A fibroblasts were incubated in the absence (−) or presence (+) of 500 ng/ml hGH at 37 °C for 15 min. Whole cell lysates were immunoprecipitated with αIRS-1 (1:250 dilution) (*lanes A* and *B*), nonimmune serum (1:125 dilution) (*lane C*), αp85 (1:100 dilution) (*lane D*), or αJAK2 (1:200 dilution) (*lanes E* and *F*). *Lane D* was loaded with one-fifth of the volume of sample loaded in the other lanes. Immunoprecipitated proteins were immunoblotted with $\alpha p85_{UBI}$ (1:2000 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migration of the 85-kDa subunit of PI 3′-kinase (p85) are indicated.

detected in cells expressing GHR_{1-318}, GHR_{1-294}, and $GHR_{\Delta P}$ (Fig. 4*A*, *lanes G–L*). In $GHR_{\Delta P}$, amino acids 297–311 of GHR, which includes the proline-rich motif (41), have been deleted. Thus, the intracellular region of GHR from the membrane to amino acid 380 and within this region, a region between 297 and 311 and another region between amino acids 318 and 380, are required for GH-mediated tyrosyl phosphorylation of IRS-1. The same regions of GHR are required for tyrosyl phosphorylation of JAK2 as indicated by the lack of GH-dependent JAK2 phosphorylation in CHO cells expressing $GHR_{\Delta P}$, GHR_{1-318}, and GHR_{1-294} (Fig. 4*B*).

The increase in basal IRS-1 tyrosyl phosphorylation in cells expressing GHR_{1-454} and GHR_{1-380} compared with GHR_{1-638} (Fig. 4*A*, *lanes A, C,* and *E*) was consistently detected. This raises the possibility that a binding site/regulatory region for a phosphatase may be present between amino acids 455 and 638 of GHR. Increased basal JAK2 activity does not appear to be

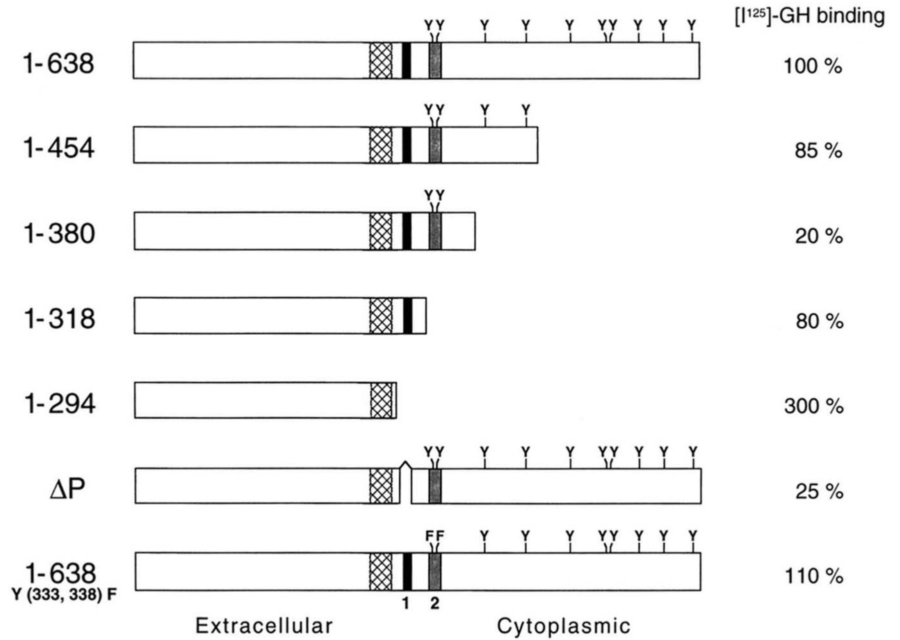

FIG. 3. **Wild-type and mutated GHR expressed in CHO cells.** The extracellular domain, transmembrane domain (*cross-hatched box*), cytoplasmic domain, homology box 1 (proline rich motif) (*solid box*), and homology box 2 (*stippled box*) of the rat liver GHR are noted. The relative ability of each cell line to bind GH is noted. Intracellular tyrosyl residues are denoted by *Y*. Tyrosines mutated to phenylalanine are denoted by *F*.

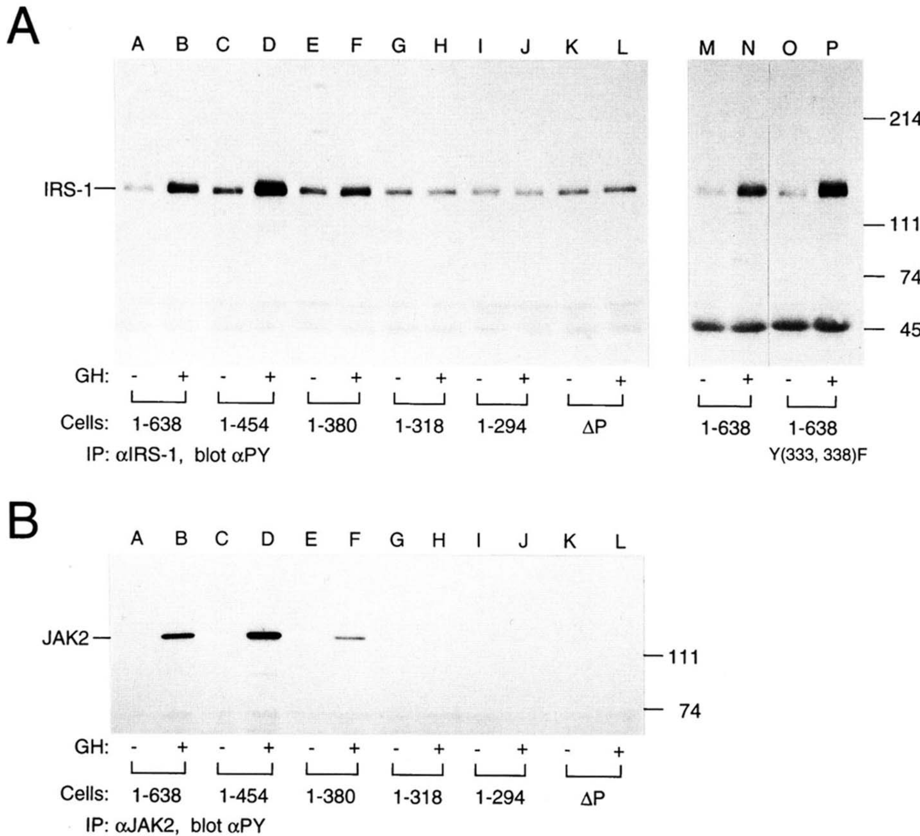

FIG. 4. **Region of GHR necessary for IRS-1 tyrosyl phosphorylation.** CHO cells expressing the indicated GHR were incubated in the absence (−) or presence (+) of 500 ng/ml hGH at 37 °C for 15 min. Whole cell lysates were immunoprecipitated with αIRS-1 (1:250 dilution) (*panel A*) or αJAK2 (1:200 dilution) (*panel B*). Immunoprecipitated proteins were immunoblotted with αPY (1:7500 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migrations of IRS-1 and JAK2 are indicated. *Lanes A–L* and *M–P* are from separate experiments.

involved since JAK2 tyrosyl phophorylation is not detected in these cell lines in the absence of GH (35).

A specific tyrosine in the receptor for insulin (42) is required for IRS-1 tyrosyl phosphorylation in response to insulin. Between amino acids 295 and 380, rat GHR contains tyrosines at amino acids 333 and 338. One or both of these tyrosines appears to be phosphorylated in response to GH.[3] To determine if these tyrosines are required for IRS-1 tyrosyl phosphorylation, the tyrosines at 333 and 338 of GHR were mutated to phenylalanines in full-length GHR_{1-638} and in GHR_{1-454}. When expressed in CHO cells, GHR_{1-638}Y(333,338)F and GHR_{1-454}Y(333,338)F mediated GH-dependent tyrosyl phosphorylation of IRS-1 (Fig. 4*A*, *lanes O* and *P*; and data not shown). Therefore, tyrosines 333 and 338 in GHR are not required for GH-dependent IRS-1 tyrosyl phosphorylation.

The region between amino acids 295 and 380 has been shown to be required for GH-dependent activation of JAK2 (35, 43, 44). Thus the role of this region of GHR in GH-dependent tyrosyl phosphorylation of IRS-1 may reside in its ability to

[3] J. VanderKuur, X. Wang, L. Zhang, G. Allevato, N. Billestrup, and C. Carter-Su, submitted for publication.

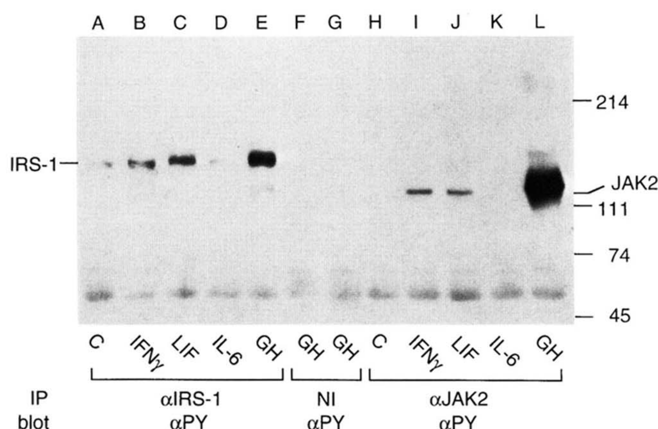

FIG. 5. **Ability of IFN-γ, LIF, and IL-6 to tyrosyl phosphorylate IRS-1.** 3T3-F442A fibroblasts were incubated with vehicle (*C*), 10 ng/ml (0.7 nM) IFN-γ, 25 ng/ml (1.3 nM) LIF, 10 ng/ml (0.5 nM) IL-6, or 500 ng/ml (23 nM) GH at 37 °C for 15 min. Whole cell lysates were immunoprecipitated with αIRS-1 (1:250 dilution) (*lanes A–E*), nonimmune serum (*lanes F* (1:250 dilution) and *G* (1:100 dilution)), or αJAK2 (1:200 dilution) (*lanes H–L*). Immunoprecipitated proteins were immunoblotted with αPY (1:7500 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migrations of IRS-1 and JAK2 are indicated. For the ligands that stimulated JAK2 tyrosyl phosphorylation, 15-min incubations and the ligand concentration used gave maximal JAK2 tyrosyl phosphorylation (data not shown).

transduce the signal that activates JAK2. Consistent with involvement of JAK2 in IRS-1 tyrosyl phosphorylation, the ability of each mutant GHR to mediate GH-dependent tyrosyl phosphorylation of JAK2 correlates with the amount of IRS-1 tyrosyl phosphorylation detected (Fig. 4). Since GH-dependent tyrosyl phosphorylation of JAK2 has been determined to reflect JAK2 kinase activity,[4] this correlation further suggests that, either directly or indirectly, stimulation of JAK2 kinase activity is required for phosphorylation of IRS-1.

Ability of IFN-γ, LIF, and IL-6 to Stimulate IRS-1 Tyrosyl Phosphorylation—If IRS-1 interacts with JAK2, one would expect other cytokines that activate JAK2 to stimulate tyrosyl phosphorylation of IRS-1 in direct relationship to their ability to activate JAK2. To determine if other cytokines, which signal via JAK2, stimulate tyrosyl phosphorylation of IRS-1, 3T3-F442A fibroblasts were treated with IFN-γ, LIF, or IL-6, IRS-1 was immunoprecipitated with αIRS-1 and immunoblotted with αPY. A ligand-dependent increase in tyrosyl phosphorylation of IRS-1 was observed in response to IFN-γ and LIF (Fig. 5, *lanes A–C*); IL-6 was not stimulatory (Fig. 5, *lanes A* and *D*). As observed with CHO cells expressing the various mutated GHR (Fig. 4), the amount of tyrosyl phosphorylation of IRS-1 detected following treatment with the various cytokines (Fig. 5, *lanes A–E*) correlates with the level of JAK2 tyrosyl phosphorylation observed (Fig. 5, *lanes H–L*).

DISCUSSION

Identification of IRS-1 as a Signaling Molecule for GH, LIF, and IFN-γ—The work presented in this paper provides strong evidence that IRS-1 is a signaling molecule for GH in 3T3-F442A fibroblasts and CHO cells expressing GHR. It supports recent findings using primary cultures of rat adipocytes published as the present report was being prepared for publication (45). Interestingly, the IRS-1 response to GH reported here for 3T3-F442A fibroblasts is much larger than that reported for adipocytes. Whether this is due to inherent differences in IRS-1 signaling at the cellular level or differences in antibody and technique remains to be determined. The ability of GH to transiently stimulate tyrosyl phosphorylation of IRS-1 and induce its association with PI 3′-kinase provides a biochemical basis for the insulin-like effects of GH observed in a variety of cell types, such as the adipocytes previously used in research with IRS-1 (45). However, GH-dependent insulin-like effects that have been characterized in the adipocyte form of 3T3-F442A cells are reduced in magnitude or undetectable in the fibroblasts form of the 3T3-F442A cells (2) used in the present study. Therefore, GH-dependent signaling through IRS-1 is likely to encompass pathways in addition to those mediating insulin-like metabolic effects.

LIF and IFN-γ, which, like GH, activate JAK2 (29, 26), have also been shown to induce the tyrosyl phosphorylation of IRS-1. IL-6 (10 ng/ml, 0.5 nM), however, failed to induce either JAK2 or IRS-1 tyrosyl phosphorylation (Fig. 5). The finding that in a sister cell line of the 3T3-F442A cells used in this study, IL-6 stimulates JAK2 and MAP kinase activity only at very high concentrations (2 μg/ml) (46) that are approximately 1000 times the reported K_d (0.3 and 8 ng/ml) for IL-6 receptors in human CESS cells (47) suggests that the inability of 10 ng/ml IL-6 to stimulate JAK2 in the present study results from the absence of high affinity IL-6 receptors in 3T3-F442A fibroblasts. IRS-1 tyrosyl phosphorylation in response to LIF, IFN-γ, and GH suggests that IRS-1 may be an element of signaling cascades for multiple members of the cytokine/hematopoietin receptor family. The presence of IRS-1 proteins in signaling pathways for members of the cytokine/hematopoietin receptor family is consistent with previous findings in FDC myeloid progenitor cells showing that IL-4, insulin, and IGF-1 stimulate tyrosyl phosphorylation of 4PS (IL-4-induced phosphotyrosine substrate), a protein antigenically related to IRS-1 that is thought to function similarly to IRS-1. IL-4 is a member of the cytokine/hematopoietin receptor family that, upon ligand binding, activates JAK3 and promotes tyrosyl phosphorylation of JAK1 (48). The finding that IRS-1 is tyrosyl phosphorylated in response to IL-4 when the common γ chain (γ_c) component of the IL-4 receptor is expressed in mouse L cells that normally lack this chain (49) suggests that IL-4 is capable of activating IRS-1. Consistent with this, when IRS-1 and the IL-4 receptor are coexpressed in 32D myeloid progenitor cells, IL-4-dependent mitogenesis (50) and activation of PI 3′-kinase and $p70^{S6}$ kinase are detected (9).

Although our results indicate that multiple ligands can utilize IRS-1 as a signaling molecule, the signaling response need not be identical for every ligand. IRS-1 contains at least 20 potential tyrosyl phosphorylation sites, at least eight of which are phosphorylated following insulin stimulation (13). Consistent with the ability of Src homology domain 2 domains in cellular proteins to bind with high affinity to phosphorylated tyrosyl residues in specific sequence motifs (51), it has been demonstrated that Src homology domain 2 domains from the 85-kDa regulatory subunit of PI 3′-kinase, GRB2, Nck, and the protein-tyrosine phosphatase SHPTP2 bind to unique residues within IRS-1 (13, 18, 15). Differences in specificity of the kinase(s) activated in response to GH *versus* insulin or IGF-1 could result in phosphorylation of unique subsets of IRS-1 tyrosyl residues and thereby alter the downstream signaling pathways activated. In the case of LIF and IFN-γ *versus* GH, all of which activate JAK2 in 3T3-F442A fibroblasts, one would expect differences in the magnitude of the signaling response due to the differences in the ability of each ligand to activate JAK2. There might also be differences in the specific response if the number of tyrosines phosphorylated differs as well.

IRS-1-mediated responses unique to a specific ligand could also arise if signaling pathways unique to that ligand acted in

[4] G. S. Campbell, E. Adkins, and C. Carter-Su, manuscript in preparation.

concert with pathways originating from IRS-1. Activation of protein kinase Cα (52) or inhibition of protein phosphatases with okadaic acid (53) has been shown to suppress tyrosyl phosphorylation of IRS-1 and decrease the binding of PI 3′-kinase to IRS-1. Therefore ligand-dependent activation of serine/threonine kinases or inhibition of serine/threonine phosphatases could suppress binding of specific signaling molecules to IRS-1. Determination of the tyrosyl residues phosphorylated in IRS-1 and of the particular proteins that bind to IRS-1 and become activated subsequent to the binding of each ligand to its receptor will begin to identify similarities and differences in cellular signaling originating from IRS-1 in response to insulin, IGF-1, and those ligands like GH that activate JAK2 kinase. Defining the molecular basis for differences in signaling by these various ligands and the basis for interactions between the cytokine network and actions regulated by insulin may identify critical metabolic steps amenable to therapeutic intervention in the management of diabetes and other metabolic diseases.

Region of GHR Required for GH-dependent Tyrosyl Phosphorylation of IRS-1—To begin to define the mechanism by which IRS-1 undergoes GH-dependent tyrosyl phosphorylation, we determined which regions of GHR are required for tyrosyl phosphorylation of IRS-1. The intracellular region of GHR from the membrane to amino acid 380 and within this region, a region between amino acids 297 and 311 containing the proline-rich motif and another region between amino acids 318 and 380, was found to be required for GH-mediated tyrosyl phosphorylation of both IRS-1 and JAK2. In this region of GHR (amino acids 295–380), homology with IFN-γ and LIF receptors is present solely in two short regions, homology box 1 (proline-rich motif, amino acids 298–305) and box 2 (amino acids 334–350) (54, 41, 55, 82). These regions of GHR are thought to be necessary for association with JAK2 and the activation of JAK2 by GH (35, 43, 44). Thus, the role of GHR in GH-dependent tyrosyl phosphorylation of IRS-1 may reside in its ability to transduce the signal, which activates JAK2 in response to GH.

The similarity in the ratio of JAK2 tyrosyl phosphorylation to IRS-1 tyrosyl phosphorylation following treatment of 3T3-F442A fibroblasts with GH, IFN-γ, and LIF and of CHO cells expressing various GHR mutants with GH provides additional evidence that IRS-1 may interact primarily with JAK2 or an accessory molecule common to these cytokine receptors that binds to JAK2. That JAK2 may contain a recognition site for IRS-1 is suggested by the fact that one of the proteins that coprecipitates with IRS-1 isolated from GH-treated cells comigrates with JAK2 precipitated using αJAK2 and vice versa (Figs. 1 and 5). The association of IRS-1 with JAK2 would make JAK2 an obvious candidate for the tyrosine kinase responsible for GH-dependent tyrosyl phosphorylation of IRS-1. This is also feasible kinetically since GH-dependent JAK2 tyrosyl phosphorylation (23, 30) precedes or is simultaneous with IRS-1 tyrosyl phosphorylation in 3T3-F442A fibroblasts (Fig. 1). If IRS-1 does in fact interact with JAK2, it would be likely that many if not all of the cytokine/hematopoietin receptors that activate JAK2 would also signal through IRS-1.

The correlation detected between JAK2 tyrosyl phosphorylation and IRS-1 tyrosyl phosphorylation is not observed with all proteins that undergo GH-dependent tyrosyl phosphorylation. In 3T3-F442A fibroblasts, GH is much more effective than IFN-γ at stimulating tyrosyl phosphorylation of JAK2 (Fig. 5). However, even though JAK2 has been shown to be required for IFN-γ-dependent activation of STAT 1 (26), tyrosyl phosphorylation and activation of STAT 1 in response to GH is actually substantially less than for IFN-γ (56, 57). The lack of correlation between JAK2 tyrosyl phosphorylation and STAT 1 tyrosyl phosphorylation following stimulation with INF-γ and GH would suggest that in contrast to IRS-1 signaling, there is some interaction between the STAT 1 signaling pathway and the receptors for the ligands responsible for STAT 1 activation.

Ligand-dependent tyrosyl phosphorylation of IRS-1 following stimulation with IGF-1, insulin, or IL-4 requires the sequence NPXY in the respective receptors (58). In rat GHR, the closest match for this sequence is NSPY and includes the tyrosine at amino acid 437. This tyrosine is not believed to be phosphorylated,[3] is not conserved in bovine, sheep, pig, and chicken GHR (59–62), and is deleted in GHR_{1-380}, which supports GH stimulation of IRS-1 tyrosyl phosphorylation (Fig. 4*A*). Thus, tyrosine 437 is not thought to be critical in interactions leading to tyrosyl phosphorylation of IRS-1. Indeed, none of the tyrosines in GHR appear necessary for IRS-1 tyrosyl phosphorylation since IRS-1 is still tyrosyl phosphorylated in CHO cells expressing GHR_{1-638}Y(333,338)F or GHR_{1-454}Y(333,338)F with the tyrosines at 333 and 338 (the only tyrosines remaining in the cytoplasmic domain of GHR_{1-380} and the only ones thought to be phosphorylated in GHR_{1-454}) mutated to phenylalanine. Nor does murine JAK1 (63), JAK2 (24), JAK3 (48), LIF receptor (64), gp130 (65), IFN-γ receptor (66), or the β-subunit of the IFN-γ receptor (67) contain the NPXY motif (82), therefore raising the possibilities that 1) in GH, LIF, and IFN-γ signaling, IRS-1 interacts with an as yet unidentified protein; 2) IRS-1 recognizes motifs in addition to the NPXY motif; or 3) the NPXY motif is actually the binding site for an auxiliary molecule, which acts as a bridge between IRS-1 and the receptors for insulin, IGF-1, and IL-4. Consistent with the latter possibility, following IL-4 stimulation of D10 T lymphocytes, 4PS has recently been shown to be present in a complex with the IL-4 receptor and the tyrosine kinase JAK1 (68). Thus, JAK1 could serve as an auxiliary molecule between the IL-4 receptor and 4PS in the IL-4 system. However, two-hybrid analysis suggests a direct interaction between insulin receptor and IRS-1 requiring the NPXY motif (69). Thus the mechanism of the JAK2-IRS-1 interaction may be distinct from that of the insulin receptor-IRS-1 interaction.

Role of PI 3′-Kinase in Cellular Signaling—PI 3′-kinase has been implicated in cellular signaling for a number of ligands that activate receptor and nonreceptor tyrosine kinases (70) including erythropoietin, IL-3, IL-5, and granulocyte-macrophage colony stimulating factor (71, 72), cytokines that induce JAK2 tyrosine kinase activity. PI 3′-kinase consists of two subunits, a regulatory 85-kDa subunit, and a catalytic 110-kDa subunit. In insulin-stimulated cells, association of PI 3′-kinase with tyrosyl phosphorylated IRS-1 activates PI 3′-kinase (73). PI 3′-kinase phosphorylates phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate at the 3′-position of the inositol ring (74). Recently PI 3′-kinase has been shown to be a dual-specificity kinase. In addition to lipid kinase activity, the activated kinase possesses serine kinase activity (75) capable of insulin-dependent serine phosphorylation of the 85-kDa subunit of PI 3′-kinase and IRS-1 (76). Autophosphorylation on serine inhibits the lipid kinase activity of PI 3′-kinase and may serve an autoregulatory role. The exact function of PI 3′-kinase in the regulation of cellular metabolism and growth is unknown. However, several lines of evidence indicate that PI 3′-kinase may regulate intracellular trafficking of proteins (77–80). GH is known to effect the translocation of GLUT 4 glucose transporters to the plasma membrane (81). Therefore, perhaps the most relevant evidence linking PI 3′-kinase and vesicular transport is the demonstration in 3T3-L1 adipocytes, a sister cell line of 3T3-F442A fibroblasts used in this report, that the PI 3′-kinase inhibitor LY294002 blocks insulin-induced glucose

transport by inhibiting insulin-stimulated GLUT 4 glucose transporter translocation (10). PI 3′-kinase activation has also been shown to be required for insulin-dependent DNA synthesis and stimulation of $p70^{S6}$ kinase, an enzyme implicated in cell cycle progression (10). Whether PI 3′-kinase is involved in GH-dependent regulation of glucose transport, $p70^{S6}$ kinase activation, stimulation of DNA synthesis, or other cellular functions awaits further investigation.

Conclusion—These results showing that GH stimulates both tyrosyl phosphorylation of IRS-1 and association of PI 3′-kinase with IRS-1 provide evidence that IRS-1 serves as a signaling molecule for GH. Furthermore, demonstration that LIF and IFN-γ, two cytokines that, like GH, activate JAK2, stimulate tyrosyl phosphorylation of IRS-1 suggests that signaling through IRS-1 may be common to multiple members of the cytokine/hematopoietin receptor family that activates JAK2. Thus at least some of the signaling pathways originating at IRS-1 are likely to be shared by these ligands. The determination that the region of GHR required for GH-mediated tyrosyl phosphorylation and activation of JAK2 is also required for the tyrosyl phosphorylation of IRS-1, in conjunction with the correlation between JAK2 tyrosyl phosphorylation and IRS-1 tyrosyl phosphorylation, suggests that IRS-1 may primarily interact with JAK2 or an auxiliary protein and at least for GHR have only minor interactions with the actual ligand receptor that mediates JAK2 activation and ultimately IRS-1 tyrosyl phosphorylation.

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