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Growth Hormone, Interferon-γ, and Leukemia Inhibitory Factor Utilize Insulin Receptor Substrate-2 in Intracellular Signaling*

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In this report, we demonstrate that insulin receptor substrate-2 (IRS-2) is tyrosyl-phosphorylated following stimulation of 3T3-F442A fibroblasts with growth hormone (GH), leukemia inhibitory factor and interferon-γ. In response to GH and leukemia inhibitory factor, IRS-2 is immediately phosphorylated, with maximal phosphorylation detected at 15 min; the signal is substantially diminished by 60 min. In response to interferon-γ, tyrosine phosphorylation of IRS-2 was prolonged, with substantial signal still detected at 60 min. Characterization of the mechanism of signaling utilized by GH indicated that tyrosine residues in GH receptor are not necessary for tyrosyl phosphorylation of IRS-2; however, the regions of GH receptor necessary for IRS-2 tyrosyl phosphorylation are the same as those required for JAK2 association and tyrosyl phosphorylation. The role of IRS-2 as a signaling molecule for GH is further demonstrated by the finding that GH stimulates association of IRS-2 with the 85-kDa regulatory subunit of phosphatidylinositol 3′-kinase and with the protein-tyrosine phosphatase SHP2. These results are consistent with the possibility that IRS-2 is a downstream signaling partner of multiple members of the cytokine family of receptors that activate JAK kinases.

IRS-1[1] is a major cytoplasmic substrate of insulin receptor (1) and several cytokine receptors that are coupled to Janus kinases. The latter group includes growth hormone (GH) receptor, which mediates tyrosine phosphorylation of IRS-1 in 3T3-F442A fibroblasts, CHO cells expressing GH receptor (GHR), and primary cultures of rat adipocytes (2–4). In addition, the 85-kDa regulatory subunit of phosphatidylinositol (PI) 3′-kinase associates with IRS-1 and becomes activated in response to GH. Tyrosyl phosphorylation of IRS-1 in response to GH suggests that GH shares some pathways utilized by insulin.

Recently, a second IRS protein was identified, which was designated IRS-2 (5). It migrates with a M_r slightly larger than IRS-1. IRS-2 has substantial structural similarity to IRS-1 with multiple potential tyrosyl phosphorylation sites, 13 of which are identical or show substantial identity to sites present in IRS-1. Conserved sites include those previously seen to bind Grb2, the protein-tyrosine phosphatase SHP2, and the 85-kDa regulatory subunit of PI 3′-kinase. IRS-2 is the dominant tyrosyl-phosphorylated protein interacting with PI 3′-kinase and Grb2 in IRS-1-deficient mice (6, 7). The ability of IRS-2 to substitute for IRS-1 is further suggested by the finding that both IRS-1 and IRS-2 mediate insulin and IL-4-stimulated mitogenesis in 32D cells (5).

In this report, we demonstrate that IRS-2 is rapidly phosphorylated on tyrosines in response to GH, LIF, and IFNγ. GH-dependent tyrosyl phosphorylation of IRS-2 requires two regions within the cytoplasmic domain of GHR; the same regions are required for JAK2 association and tyrosyl phosphorylation (3, 8, 9). GH is also shown to promote association of IRS-2 with both PI 3′-kinase and SHP2. This and recent reports of IRS-2 tyrosyl phosphorylation in response to IL-2, IL-4, and IL-7 (cytokines that activate JAK1 and JAK3; Ref. 10), and IFNα (a cytokine that activates JAK1 and Tyk2; Ref. 11) suggest that signaling through IRS-2 may be a common element in signaling for many members of the cytokine family of receptors that activate JAK tyrosine kinases.

EXPERIMENTAL PROCEDURES

Materials—Recombinant 22,000-dalton hGH and porcine insulin were a gift of Lilly. Human recombinant IGF-1 was from Calbiochem. Murine recombinant LIF was from R & D Systems. Murine recombinant IFNγ and chicken egg ovalbumin were from Sigma. 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 (CRG-7) was from Intergen. Prestained molecular weight standards were from Life Technologies, Inc. Nitrocellulose paper was from Schleicher & Schuell. The enhanced chemiluminescence (ECL) detection system was from Amersham. X-ray film was from DuPont.

Antisera—Anti-phosphotyrosine binding antibody (αTyr(P)) (4G10), and antiserum to the 85-kDa subunit of PI 3′-kinase (for immunoprecipitation 05-212, for blotting 06-195) (αp85) and SHP2 (06-118) (αSHP2) were purchased from Upstate Biotechnology, Inc. Antibody to IRS-1 (αIRS-1) was prepared against recombinant IRS-1 (12). Antibody to IRS-2 (αIRS-2) was prepared in rabbits to a glutathione *S*-transferase fusion protein containing amino acids 619–746 of murine IRS-2 (5). Antibody to JAK2 (αJAK2) was prepared against a synthetic peptide corresponding to amino acids 758–776 of murine JAK2 (13).

Mutagenesis, Transfection, and Cell Culture—CHO cells expressing full-length rat GHR or GHR mutated to replace lysine codons 455, 381, 319, or 295 with termination codons; or to delete the codons for amino

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[1] The abbreviations used are: IRS, insulin receptor substrate; CHO, Chinese hamster ovary; GH, growth hormone; hGH, human growth hormone; GHR, growth hormone receptor; IGF-1, insulin-like growth factor-1; IFNγ, interferon-γ; IL, interleukin; LIF, leukemia inhibitory factor; PI 3′-kinase, phosphatidylinositol 3′-kinase; SHP2, Src homology 2 domain-containing protein-tyrosine phosphatase 2; MAP, mitogen-activated protein.

acids 297–311; or to replace tyrosine codons 333 and 338 with codons for phenylalanine have been described previously (3). Amino acids in GHR are numbered according to Ref. 14. The binding affinity of each cell line for hGH was similar (15, 16).[2] The relative ^{125}I-GH binding by CHO cell lines expressing various GHRs was GHR-1–638 (wild type), 100%; GHR1–454, 130%; GHR1–380, 70%; GHR1–318, 60%; GHR1–294, 280%; and GHRΔP, 60%; GHR1–454Y(333, 338)F, 90%. The stock of 3T3-F442A cells was a kind gift of H. Green (Harvard University, Boston, MA). Conditions used to culture CHO and 3T3-F442A cells have been described previously (3).

Immunoprecipitation and Immunoblotting—Confluent cells were incubated in serum-free medium overnight (16–20 h) as described previously (17). Cells were incubated at 37 °C with hGH, cytokine, or growth factor at the indicated concentrations for various time intervals and then rinsed three times with ice-cold PBSV (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4). Cells were solubilized 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), and 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 three times with 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 resolved by SDS-polyacrylamide gel electrophoresis (30:0.5, acrylamide:bisacrylamide) on 5–12% gradient polyacrylamide gels (when visualizing IRS-2 and PI 3′-kinase) or 8% gels (for SHP2). For experiments using CHO cells, the protein content of the cell supernatant was determined and used to normalize the amount of sample added to each lane. When visualizing IRS-2, 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. When visualizing PI 3′-kinase and SHP2, the SDS was omitted and proteins transferred for 1 h. Blots were incubated with the appropriate antibody and visualized by ECL detection as described previously (17). To reprobe a 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 verify that all primary antibody had been removed, and then reprobed using the second antibody.

For quantification, autoradiographs were scanned using an Agfa ArcusII scanner and Fotolook SA software (Mortsel, Belgium). The resulting image was analyzed using Molecular Analyst image analysis software from Bio-Rad. Ligand-dependent tyrosyl phosphorylation of IRS-2, and association of the 85-kDa subunit of PI 3′ kinase or SHP2 with IRS-2 is compared to treatment with vehicle. Means and standard errors (S.E.) of independent experiments are reported. Tyrosyl phosphorylation of IRS-2 in CHO cells expressing various GHR mutants is compared to stimulation observed with vehicle alone in cells expressing GHR1–638. One-tailed paired Student's *t* tests were used to assess statistical significance.

Homology Searches—Homology searches were performed using the EditSeq program from DNAstar, Inc.

RESULTS

Ability of GH, IFNγ, and LIF to Stimulate IRS-2 Tyrosyl Phosphorylation—Signaling utilizing IRS-2 appears to be dependent upon its tyrosyl phosphorylation (6, 7). To determine if IRS-2 is tyrosyl-phosphorylated in response to GH, 3T3-F442A fibroblasts were incubated for 15 min with different concentrations of GH. Cellular proteins were solubilized, immunoprecipitated with αIRS-2, and immunoblotted with αTyr(P). Concentrations of GH as low as 5 ng/ml (0.23 nM) induced tyrosyl phosphorylation of a protein with a M_r (180,000–190,000) appropriate for IRS-2 (Fig. 1, *lanes B–F*). At 500 ng/ml GH, tyrosyl phosphorylation of this protein is 4.2 times (S.E. = 0.5, n = 5) that seen in vehicle-treated cells. Consistent with this phosphoprotein being IRS-2, it co-migrates with a protein in the immunoprecipitate that is recognized by αIRS-2 in immunoblots (Fig. 1, *lane A*), co-migrates with IRS-2 phosphorylated in response to IGF-1 and insulin (Fig. 1, *lanes G* and *H*), and migrates as a slightly larger protein than IRS-1 (Fig. 1, *lane I*).

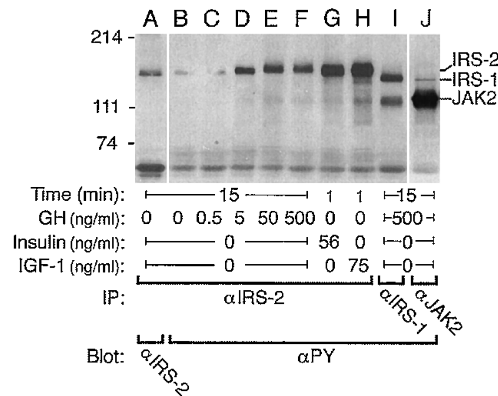

FIG. 1. **GH induces tyrosyl phosphorylation of IRS-2.** 3T3-F442A fibroblasts were incubated at 37 °C with vehicle (*lanes A* and *B*) or the indicated concentrations of hGH (*lanes C–F*, *I*, and *J*) for 15 min, or 56 ng/ml (10 nM) insulin (*lane G*) or 75 ng/ml (10 nM) IGF-1 (*lane H*) for 1 min. Cell lysates were immunoprecipitated with αIRS-2 (1:250 dilution) (*lanes A–H*), αIRS-1 (1:250 dilution) (*lane I*), or αJAK2 (1:200 dilution) (*lane J*). Immunoprecipitated proteins were immunoblotted with αTyr(P) (1:7500 dilution) (*lanes B–J*). For *lane A* the blot was stripped and reprobed with αIRS-2 (1:2000 dilution). The molecular weight (×10^{-3}) of protein standards and the migration of IRS-2, IRS-1, and JAK2 are indicated.

To examine whether other cytokines that activate JAK2 stimulate tyrosyl phosphorylation of IRS-2, 3T3-F442A fibroblasts were treated with GH, IFNγ, or LIF at concentrations determined, in experiments not shown, to be sufficient to yield maximal tyrosyl phosphorylation of JAK2 at 15 min. Ligand-dependent tyrosyl phosphorylation of IRS-2 is detected in response to IFNγ and LIF, as well as to GH (Fig. 2*A*). However, tyrosyl phosphorylation of IRS-2 following treatment with IFNγ or LIF is substantially less than that following stimulation with GH. Differences in the time course for IRS-2 phosphorylation by the different ligands are also detected. In response to GH and LIF, maximal signal is detected at 15 min. For LIF the signal is 2.5 times (S.E. = 0.6, n = 3) and for GH 4.2 times (S.E. = 0.5, n = 5) the signal detected with vehicle alone. The signal is diminished by 60 min (Fig. 2, *B* and *C*). In contrast, with IFN-γ onset of IRS-2 tyrosyl phosphorylation is slower and the signal is more prolonged, at 15 min the signal is 1.9 times (S.E. = 0.4, n = 3), and at 60 min 2.2 times (range = 0.7, n = 2) the signal with vehicle alone (Fig. 2*D*). For comparison, in response to insulin, by 1 min tyrosyl phosphorylation of IRS-2 is 15 times (S.E. = 4.6, n = 3) the signal with vehicle alone, and present at approximately 50% of this level from 5 to 60 min (Fig. 2*E*).

To determine if differences in the magnitude of IRS-2 tyrosyl phosphorylation correlate with tyrosyl phosphorylation of JAK2 induced by GH, LIF, and IFNγ, ligand-dependent tyrosyl phosphorylation of JAK2 was monitored by immunoprecipitating with αJAK2 and immunoblotting with αTyr(P) (Fig. 2*A*, *lanes E–H*; see also *lanes G–L* of *panels B–D*). The relative magnitude and time course of IRS-2 tyrosyl phosphorylation reflected the JAK2 tyrosyl phosphorylation induced by each ligand. This correlation suggests that JAK2 activation may be necessary for IRS-2 tyrosyl phosphorylation. Similarly, following insulin stimulation, tyrosyl phosphorylation of IRS-2 correlates with tyrosyl phosphorylation of insulin receptor (Fig. 2*E*). Whether ligand-dependent differences in the extent of tyrosyl phosphorylation of IRS-2 and JAK2 result from intrinsic differences in the responses elicited by each ligand or reflect differences in the number of GH, LIF, and IFNγ receptors expressed in 3T3-F442A fibroblasts is not known. However, consistent with the results in 3T3-F442A fibroblasts, in IM-9 lymphocytes, rapid transient stimulation of JAK2 tyrosyl phos-

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

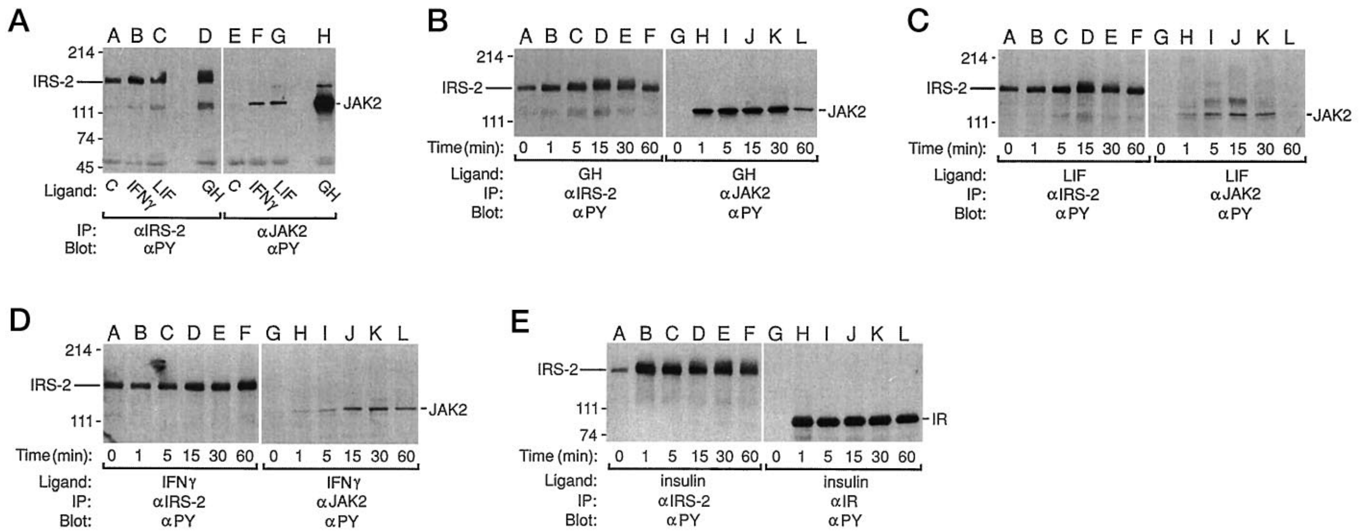

FIG. 2. **Time course of GH, LIF, IFNγ, and insulin induced tyrosyl phosphorylation of IRS-2.** *Panel A*, 3T3-F442A fibroblasts were incubated at 37 °C for 15 min with vehicle (*C*) (*lanes A* and *E*), 10 ng/ml (0.7 nM) IFNγ (*lanes B* and *F*), 25 ng/ml (1.3 nM) LIF (*lanes C* and *G*), or 500 ng/ml (23 nM) GH (*lanes D* and *H*). Cell lysates were immunoprecipitated with αIRS-2 (1:250 dilution) (*lanes A–D*) or αJAK2 (1:200 dilution) (*lanes E–H*). Immunoprecipitated proteins were immunoblotted with αTyr(P) (1:7500 dilution). *Panels B–E*, 3T3-F442A fibroblasts were incubated with: *B*, 500 ng/ml (23 nM) hGH; *C*, 10 ng/ml (0.7 nM) IFNγ; *D*, 25 ng/ml (1.3 nM) LIF; *E*, 28 ng/ml (5 nM) insulin at 37 °C for the indicated times. Cell lysates were immunoprecipitated with αIRS-2 (1:250 dilution) (*lanes A–F*) or αJAK2 (1:200 dilution) (*lanes G–L*). Immunoprecipitated proteins were immunoblotted with αTyr(P) (1:7500 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migrations of IRS-2, JAK2, and insulin receptor (*IR*) are indicated. The concentrations of GH, IFNγ, and LIF used were sufficient to yield maximal JAK2 tyrosyl phosphorylation at 15 min (data not shown).

phorylation is detected in response to GH, while prolonged JAK2 tyrosyl phosphorylation (essentially equivalent at 10 and 60 min) is observed in response to IFNγ (18). Thus, substantial temporal differences in activation and deactivation of JAK2 and IRS-2 in response to GH, LIF, and INFγ appear to be present.

Following stimulation by GH, LIF, and insulin, IRS-2 migrates as a broader band at the later time points (Fig. 2, *B*, *C*, and *E*). This effect is not observed with IFNγ (Fig. 2*D*). Broadening of the IRS-2 band was also observed to be dependent upon the concentration of GH, with broadening observed only at higher concentrations (Fig. 1). Since a decrease in the mobility of a protein is often associated with increased protein phosphorylation, ligand-dependent differences in IRS-2 mobility suggest that individual phosphorylation sites (serine, threonine, or tyrosine) in IRS-2 may be phosphorylated to varying degrees, in response to the different ligands.

In addition to IRS-2, tyrosyl-phosphorylated proteins with M_r = 120,000–130,000 are also detected in αIRS-2 immunoprecipitates from LIF and GH-treated cells (Fig. 1, *lanes E* and *F*; Fig. 2, *A–C*, *lanes C* and *D*). The lower portion of the 120–130-kDa band is also detected following IGF-1 treatment (Fig. 1, *lane H*). Coprecipitation of a tyrosyl-phosphorylated protein with this M_r has previously been observed in rat FAO hepatoma cells following insulin treatment (5). This protein could be Gab1, a protein with homology to IRS-1, especially in the pleckstrin homology domain. Gab1 was recently shown to undergo insulin-dependent tyrosyl phosphorylation in A431 cells and to bind Grb2, phospholipase Cγ, PI 3′-kinase, and SHP2 (19). Following GH (but not IGF-1 or insulin) stimulation, the upper portion of the 120–130-kDa band contains a distinct protein which co-migrates with JAK2; however, insufficient protein is co-precipitated to permit identification by immunoblotting with αJAK2. The inability to detect this protein following treatment with LIF and IFNγ is thought to be due to the smaller amount of IRS-2 that is phosphorylated. The ability of JAK2 to associate with IRS-2 in response to GH was also tested by immunoprecipitating with αJAK2. Only protein that comigrates with IRS-1 is detected; no IRS-2 is detected (Fig. 1, *lane J*). These results suggest that IRS-2 may be present in a complex with JAK2. However, it appears that less IRS-2 than IRS-1 is present and/or the affinity of JAK2 for IRS-2 is less than for IRS-1.

Ability of GH to Induce Association of PI 3′-Kinase with IRS-2—IRS-1, IRS-2, and JAK2 each contain the Y*XX*M motif preferentially recognized by the Src homology 2 domains of the 85-kDa regulatory subunit of PI 3′-kinase (p85) (5, 13, 20). Furthermore, p85 binds to IRS-2 and is activated following insulin stimulation (6, 7). To determine if GH promotes association of p85 with IRS-2 or with JAK2, solubilized 3T3-F442A fibroblasts were immunoprecipitated with αIRS-2 or αJAK2 and immunoblotted with αp85. Following GH treatment, the amount of 85-kDa subunit of PI 3′-kinase that co-precipitates with IRS-2 is 2.1 times (S.E. = 0.2, n = 4) the level detected following treatment with vehicle alone (Fig. 3*A*, *lanes A* and *B*). This protein is identified by αp85 in immunoblots (Fig. 3*A*, *lane D*). In contrast, p85 does not appear to associate directly with JAK2, since p85 was not detected in αJAK2 immunoprecipitates of GH-treated cells (Fig. 3*A*, *lanes E* and *F*). No p85 is detected with nonimmune serum (Fig. 3*A*, *lane C*). When proteins from GH-treated cells were immunoprecipitated with αp85 and immunoblotted with αTyr(P), tyrosyl-phosphorylated proteins that co-migrate with IRS-2, IRS-1, GHR, and JAK2 are detected (Fig. 3*B*). Thus, it appears that PI 3′-kinase is present in a multiprotein complex containing GHR, JAK2, IRS-1, and IRS-2; however, PI 3′-kinase appears to associate directly with IRS-2. This is consistent with the previous finding that PI 3′-kinase directly associates with IRS-1 (3).

Ability of GH to Induce Association of SHP2 with IRS-2—Binding sites for SHP2 present in IRS-1 are conserved in IRS-2 (5). To determine if GH can induce association of SHP2 with IRS-2, solubilized 3T3-F442A fibroblasts were immunoprecipitated with αIRS-2 and immunoblotted with αSHP2. Following GH treatment, the amount of SHP2 that specifically co-precipitates with IRS-2 is 2.9 times (S.E. = 0.1, n = 3) the level with vehicle alone (Fig. 4, *lanes A* and *B*). Thus, GH stimulates association of SHP2 with IRS-2 in these cells.

Identification of the Region of GHR Necessary for IRS-2

FIG. 3. **GH-dependent association of the 85-kDa regulatory subunit of PI 3′-kinase with IRS-2.** *Panel A*, 3T3-F442A fibroblasts were incubated at 37 °C for 15 min in the absence (−) (*lanes A* and *E*) or presence (+) (*lanes B–D* and *F*) of 500 ng/ml hGH. Cell lysates were immunoprecipitated with αIRS-2 (1:250 dilution) (*lanes A* and *B*), nonimmune serum (1:200 dilution) (*lane C*), αp85 (1:200 dilution) (*lane D*), or αJAK2 (1:200 dilution) (*lanes E* and *F*). Imunoprecipitated proteins were immunoblotted with αp85 (1:2000 dilution). *Panel B*, 3T3-F442A fibroblasts were incubated at 37 °C for 15 min in the absence (−) (*lanes A*, *C*, and *E*) or presence (+) (*lanes B*, *D*, and *F*) of 500 ng/ml hGH. Cell lysates were immunoprecipitated with αIRS-1 (1:250 dilution) (*lanes A* and *B*), αIRS-2 (1:250 dilution) (lanes C-D), or αp85 (1:200 dilution) (*lanes E* and *F*). Imunoprecipitated proteins were immunoblotted with αTyr(P) (1:7500 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migration of p85, IRS-2, IRS-1, JAK2, and GHR are indicated.

FIG. 4. **GH-dependent association of SHP2 with IRS-2.** 3T3-F442A fibroblasts were incubated at 37 °C with vehicle (−) (*lanes A* and *D*) for 15 min, 500 ng/ml (23 nM) hGH (*GH*) (*lanes B*, *E*, and *G*) for 15 min, or 28 ng/ml (5 nM) insulin for 5 min (*lanes C* and *F*). Cell lysates were immunoprecipitated with αIRS-2 (1:250 dilution) (*lanes A–C*), nonimmune serum (1:200 dilution) (*lanes D–F*), or αSHP2 (1:100 dilution) (*lane G*). Immunoprecipitated proteins were immunoblotted with αSHP2 (1:1000 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migration of SHP2 are indicated.

FIG. 5. **Wild-type and mutated rat 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. Intracellular tyrosyl residues are denoted by *Y*. Tyrosines mutated to phenylalanine are denoted by *F*.

Tyrosyl Phosphorylation—To begin to understand the molecular basis for the interaction between IRS-2 and GHR, the ability of mutated GHR to stimulate tyrosyl phosphorylation of IRS-2 was evaluated (mutants summarized in Fig. 5). CHO cells expressing the respective GHR mutants were solubilized, IRS-2 was immunoprecipitated using αIRS-2 and immunoblotted with αTyr(P). GHR1–638 (wild-type), GHR1–454, and to a lesser extent GHR1–380 promote a GH-dependent increase in IRS-2 tyrosyl phosphorylation (Fig. 6, *A* and *B*, *lanes A–F*). No GH-dependent tyrosyl phosphorylation of IRS-2 is detected in cells expressing GHR1–318, GHR1–294, and GHRΔP (Fig. 6, *A* and *B*, *lanes G–L*). In GHRΔP, amino acids 297–311 of GHR have been deleted, which removes the proline-rich box 1 motif that is conserved in many members of the cytokine family of receptors (21). Thus, two regions in the cytoplasmic domain of GHR appear to be required for GH-mediated tyrosyl phosphorylation of IRS-2, one between amino acids 297 and 311 and another between amino acids 318 and 380.

The increase in basal IRS-2 tyrosyl phosphorylation in cells expressing GHR1–454 and GHR1–380 compared to GHR1–638 (Fig. 6, *A* and *B*, *lanes C* and *E*) was consistently detected. A similar effect was seen with IRS-1 (3). Increased basal JAK2 activity does not appear to be involved, since in these cell lines, JAK2 tyrosyl phosphorylation is not detected in the absence of GH (8). This raises the possibility that a binding site/regulatory region for a phosphatase may be present between amino acids 455 and 638 of GHR.

A tyrosine in the context of an NP*X*Y motif in the receptors for insulin, IGF-1, and IL-4 is required for tyrosyl phosphorylation of IRS-1 in response to these ligands (22–25). When phosphorylated, the NP*X*Y motif of these receptors interacts with the phosphotyrosine-binding domain in IRS-1 (IH-2^{PTB}). This domain is highly conserved between IRS-1 and IRS-2 (5). Thus, ligand-dependent tyrosyl phosphorylation of either the ligand receptor or an associated molecule is thought to be required for interaction with IRS-2. Between amino acids 295 and 380, the region of GHR required for GH-dependent IRS-2 tyrosyl phosphorylation, rat GHR contains tyrosines at amino acids 333 and 338. One or both of these tyrosines appear to be phosphorylated in response to GH (26). To determine if these tyrosines are required for IRS-2 tyrosyl phosphorylation, tyrosines 333 and 338 of GHR were mutated to phenylalanines in GHR1–454. The remaining two tyrosines in GHR1–454 do not appear to be tyrosyl-phosphorylated (26). When expressed in CHO cells, GHR1–454Y(333,338)F mediates GH-dependent tyrosyl phosphorylation of IRS-2 (Fig. 6, *panel A*, *lanes O* and *P*; and *panel B*, *lanes M* and *N*). Therefore, phosphorylation of tyrosines in GHR does not appear to be required for GH-dependent tyrosyl phosphorylation of IRS-2.

The proline-rich box 1 motif and the region between amino acids 318 and 380 in the cytoplasmic domain of GHR required for IRS-2 phosphorylation are also required for GH-dependent tyrosyl phosphorylation of JAK2 and JAK2 association with GHR (Fig. 6) (8, 9, 27). Thus, the function of GHR in mediating GH-dependent IRS-2 tyrosyl phosphorylation may be to trans-

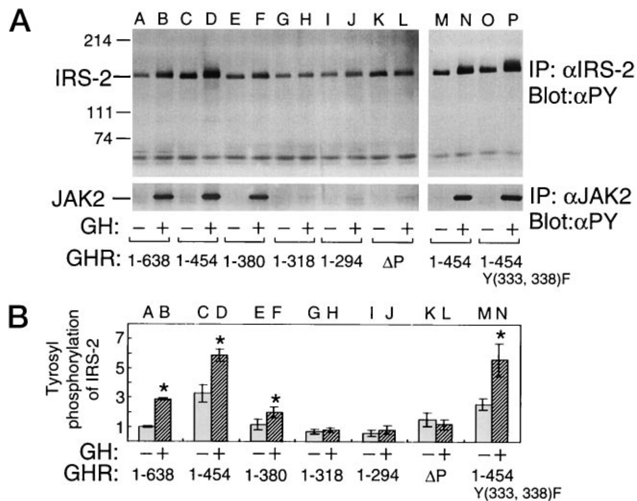

FIG. 6. **Region of GHR necessary for tyrosyl phosphorylation of IRS-2.** *Panel A*, CHO cells expressing the indicated GHR were incubated in the absence (−) or presence (+) of 500 ng/ml hGH at 37 °C for 15 min. Cell lysates were immunoprecipitated with αIRS-2 (1:250 dilution) or αJAK2 (1:200 dilution) as indicated. Immunoprecipitated proteins were immunoblotted with αTyr(P) (1:7500 dilution). The molecular weight ($\times 10^{-3}$) of protein standards and the migration of IRS-2 and JAK2 are indicated. *Lanes A–L* and *M–P* are from separate experiments. *Panel B*, levels of tyrosyl phosphorylation of IRS-2 in CHO cells expressing the indicated GHR were quantified and normalized to vehicle-treated cells expressing GHR1-638. The data are expressed as the mean ± range (n = 2) for GHR1–638, GHR1–454, and GHR1–454Y(333,338)F; or as the mean ± S.E. (n = 3) for GHR1–380, GHR1–294, and GHRΔP. For each transfected GHR, one-tailed paired t tests were used to assess whether vehicle and GH-treated values were statistically significant. Significant differences ($p < 0.05$) are denoted with an *asterisk* (*).

duce the signal that activates JAK2. Consistent with IRS-2 interacting with either tyrosyl-phosphorylated JAK2 or a molecule associated with tyrosyl-phosphorylated JAK2, the ability of each mutant GHR to mediate GH-dependent tyrosyl phosphorylation of IRS-2 correlates with the amount of JAK2 tyrosyl phosphorylation detected (Fig. 6*A*). Since GH-dependent tyrosyl phosphorylation of JAK2 reflects JAK2 kinase activity,[3] this correlation further suggests that, either directly or indirectly, JAK2 kinase activity is required for IRS-2 phosphorylation.

DISCUSSION

Work presented in this paper using 3T3-F442A fibroblasts and CHO cells expressing GHR provides strong evidence that IRS-2 is a component of GH signaling. Thus both IRS family members identified to date, IRS-1 (2–4) and IRS-2, have a role in GH signaling. Clearly, there is some overlap in function between IRS-2 and IRS-1. Both IRS-1 and IRS-2 bind PI 3′-kinase in response to GH (Refs. 3 and 4 and this paper). Furthermore, 13 of 22 potential tyrosine phosphorylation sites within IRS-2 are the same as or similar to sites in IRS-1 (5). Signaling molecules that bind to these shared motifs are likely to be signaling molecules for both IRS-1 and IRS-2. No downstream signaling molecules unique to either IRS-1 or IRS-2 have yet been identified. However, potential for such molecules exists; IRS-2 contains nine potential phosphorylation sites not shared with IRS-1 and IRS-1 contains five sites not shared with IRS-2 (5). Distinct roles for IRS-1 and IRS-2 in GH signaling could also arise from differences in tissue expression of IRS-1 and IRS-2 (5).

We and others have shown that, in addition to GHR, other members of the cytokine receptor family induce tyrosyl phosphorylation of IRS-2. These include receptors for LIF and IFNγ (this paper); IL-2, IL-4, and IL-7 (10, 28); and IFNα (11). These receptors activate various members of the JAK kinase family; thus, activation of IRS-2 does not appear to be limited to a specific JAK. Whether these receptors initiate the same downstream signaling pathways as the receptors for insulin IGF-1, GH or each other is not known. However, it seems reasonable to speculate that different tyrosine kinases will have differing specificities for the 22 tyrosines in IRS-2. In support of this, the receptors for insulin and IGF-1 have varying specificity for individual tyrosines in IRS-1, at least *in vitro* (29). Furthermore, in response to GH, IFN-γ, and LIF, there appear to be differences in the extent of phosphorylation of individual IRS-2 molecules. Thus, different subsets of signaling molecules might be recruited by IRS-2 in response to different ligands. Dose and length of exposure to ligand would also affect the degree of phosphorylation and could therefore potentially affect which signaling molecules are recruited to IRS-2.

The ability of multiple members of the cytokine receptor family to induce tyrosyl phosphorylation of IRS-2 suggests that a region(s) of homology essential for IRS-2 tyrosyl phosphorylation is present in these receptors. Within the cytoplasmic domain of receptors in the cytokine receptor family, two motifs have been recognized, proline-rich box 1 and box 2 (21, 30, 31). These motifs, reported to be required for recognition of the appropriate JAK kinase by these receptors (8, 9, 27, 32–37), are present in the regions of GHR required for GH-dependent tyrosyl phosphorylation of IRS-2. This suggests that JAK2 may be responsible for phosphorylating IRS-2. The finding that additional regions of GHR do not appear to be required for IRS-2 phosphorylation suggests that IRS-2 may interact directly with JAK2, rather than with GHR. Consistent with the ability of JAK kinases to associate directly with and phosphorylate IRS proteins, when IRS-2 is coexpressed with JAK1 or JAK3 in COS-7 cells, IRS-2 is tyrosyl-phosphorylated and coprecipitates with tyrosyl-phosphorylated JAK1 or JAK3 (10). Furthermore, a complex between IRS-2 and JAK1 has been detected in human T lymphocytes (constitutive) and D10G4.1 T helper lymphocytes (ligand-dependent) (10, 38). A complex between IRS-2 and Tyk2 has been detected in human acute erythroleukemia (HEL) cells (11). Also consistent with a protein other than GHR, such as the Janus kinases, providing a binding site for IRS-2, is the finding that no phosphorylated tyrosines in GHR appear to be required for IRS-2 phosphorylation in response to GH.

The question of whether phosphorylation of IRS-2 requires binding of IRS-2 to a site within GHR, JAK2, or an accessory protein is an intriguing one. Phosphorylation of IRS-1 in response to IL-4, insulin, and IGF-1 appears to require interaction between the phosphotyrosine binding domain of IRS-1 and a tyrosyl-phosphorylated NP*X*Y motif in the ligand receptor (22, 23, 25). Since a domain homologous to the phosphotyrosine binding domain of IRS-1 is present in IRS-2 (5), IRS-2 has been hypothesized to interact with the NP*X*Y motif in the receptors for IL-4, insulin, and IGF-1. However, GHR and JAK2 do not contain NP*X*Y motifs (13, 14). Furthermore, no phosphorylated tyrosines within GHR appear to be required for GH-dependent tyrosyl phosphorylation of IRS-2. Thus, as hypothesized for IRS-1 (3), IRS-2 may primarily interact with an auxiliary protein in the GHR·JAK2 complex via an NP*X*pY motif and have only minor, interactions with GHR or JAK2. Alternatively, there may be several domains in IRS-2 that interact with ligand-bound receptor complexes. Some of these domains may bind motifs other than NP*X*pY and may in fact bind motifs that do not contain phosphorylated tyrosines. Consistent with IRS-2

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

associating with proteins by multiple mechanisms, glutathione *S*-transferase fusion proteins containing the pleckstrin homology domain of IRS-1 or IRS-2 precipitate Tyk2 from extracts of IFNα-treated human multiple myeloma (U266) cells (11). Consistent with IRS-2 interacting with an auxiliary protein other than JAK2, in U266 cells, IFNγ stimulates tyrosyl phosphorylation of JAK2 but does not induce tyrosyl phosphorylation of IRS-2 (11). Perhaps 3T3-F442A fibroblasts, in which IFNγ does induce IRS-2 tyrosyl phosphorylation, have a signaling molecule that is not present in U266 cells.

The binding of the 85-kDa regulatory subunit of PI 3′-kinase to IRS-2 has been shown to activate PI 3′-kinase (5, 6). Thus, the ability of GH to recruit p85 to IRS-2 suggests that GH activates PI 3′-kinase. Potential roles for PI 3′-kinase in GH action include regulation of glucose transport, nuclear translocation of MAP kinases, and regulation of protein kinase C. Involvement of PI 3′-kinase in GH-dependent glucose transport is suggested by the finding in rat adipocytes that GH-dependent lipid synthesis, which is metabolically downstream from glucose transport, is blocked by wortmannin, an inhibitor of PI 3′-kinase (39). GH, like insulin, stimulates glucose transport at least in part by inducing translocation of GLUT4 from low density microsomes to the plasma membrane (40).[4] Wortmannin blocks insulin-stimulated translocation of GLUT4 glucose transporters from low density microsomes to the plasma membrane (41).

Involvement of PI 3′-kinase in nuclear translocation of the MAP kinase ERK1 is suggested by recent studies using wortmannin as well as a mutant polyomavirus middle-T tumor antigen defective in the ability to bind PI 3′-kinase (42). ERK1 and ERK2 are activated by GH (43–45); therefore, PI 3′-kinase may also be involved in GH-dependent activation of genes such as c-*fos* (46–48) that are thought to require MAP kinase for transcriptional activation (49–51).

Phosphatidylinositol 3,4-P_2 and phosphatidylinositol 3,4,5-P_3, products of PI 3′-kinase, have recently been shown to activate Ca^{2+}-independent protein kinase C isoforms γ, ϵ, and η (52). The findings that GH induces translocation of the ϵ isoform of protein kinase C from the cytoplasm to the plasma membrane in 3T3-F442A fibroblasts (53) and that the PI 3′-kinase inhibitor wortmannin inhibits GH-dependent diacylglycerol formation in rat adipocytes (54), raise the possibility that in some cell types, PI 3′-kinase may be involved in GH-dependent activation of protein kinase C.

The role of SHP2 in GH signaling is presently unknown. It seems unlikely that SHP2 bound to IRS-2 is the primary phosphatase responsible for dephosphorylating JAK2 and/or GHR. Overexpression of a catalytically inactive form of SHP2 blocks, rather than stimulates, induction by prolactin of a β-casein reporter gene, a JAK2- and prolactin-dependent event (55). It seems more likely that functions regulated by SHP2 as a result of GH activation include the same functions regulated by SHP2 when activated by insulin. SHP2 has been implicated in insulin, IGF-1, and epidermal growth factor-dependent stimulation of Ras, MAP kinase, DNA synthesis, and c-*fos* reporter gene expression (56–59). These responses are blocked by overexpression of a catalytically inactive form of SHP2, suggesting that SHP2 is a positive regulator of these functions. Presumably some of these or other not yet identified effects are due to increased phosphatase activity. However, SHP2 has also been reported to function as an adapter for proteins such as Grb2 (60–62). Consistent with SHP2 serving as an adapter protein for IRS-1 and IRS-2, in baby hamster kidney cells stably expressing insulin receptor and transiently overexpressing IRS-1, overexpression of SHP2 increases the amount of IRS-1 associated with insulin receptor (63). In addition, overexpression of SHP2 (either wild-type or catalytically inactive) increases insulin-dependent glucose transport in cells transiently overexpressing IRS-1 (63). Since the MAP kinase cascade, c-*fos* gene expression, and glucose transport are also regulated by GH, it seems likely that the SHP2 which binds to IRS-2 in response to GH may play a role in the regulation of these functions by GH.

CONCLUSIONS

These results showing that GH promotes tyrosyl phosphorylation of IRS-2 and association of SHP2 and the 85-kDa regulatory subunit of PI 3′-kinase with IRS-2 provide evidence that IRS-2 has a role in GH signaling. GH-dependent association of SHP2 with IRS-2 identifies a potential mechanism for GH-dependent activation of this phosphatase. The finding that the region of GHR required for IRS-2 tyrosyl phosphorylation is the same region required for JAK2 association and activation, and that phosphotyrosines in GHR do not appear to be required for GH-dependent tyrosyl phosphorylation of IRS-2, suggests that interaction of IRS-2 with the GHR-JAK2 complex may occur principally through JAK2 or an auxiliary molecule associated with JAK2. The abilities of LIF and IFNγ to also stimulate tyrosyl phosphorylation of IRS-2 suggest that signaling through IRS-2 may be common to many members of the cytokine receptor family. However, differences in duration and magnitude of IRS-2 tyrosyl phosphorylation in response to GH, LIF, IFNγ, and insulin suggest that the ultimate biologic responses culminating from signaling through IRS-2 could differ for the different ligands.

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