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Interferon-α Engages the Insulin Receptor Substrate-1 to Associate with the Phosphatidylinositol 3′-Kinase*

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Interferon-α (IFNα) induces rapid tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), a docking protein with multiple tyrosine phosphorylation sites that bind to the Src homology 2 (SH2) domains of various signaling proteins. During IFNα stimulation, the p85 regulatory subunit of the phosphatidylinositol 3′-kinase binds via its SH2 domains to tyrosine-phosphorylated IRS-1, and phosphatidylinositol 3′-kinase activity is detected in association with IRS-1. Thus, IFNα responses occur by activation of the IRS signaling system, which it shares with insulin, insulin-like growth factor-1, and interleukin-4.

The Type I interferons (IFNα, IFNβ, and IFNω)[1] exert a variety of biological effects on normal and neoplastic cells that include antiviral and antiproliferative activities (1). Immediately after IFNα stimulation, several signaling proteins in the receptor complex become tyrosine-phosphorylated, including the α and β subunits of the Type I IFN receptor (2, 3) and the Tyk-2 and Jak-1 tyrosine kinases (4–7). Both kinases associate with components of the Type I IFN receptor (7, 8), and their activation early in the IFNα signaling cascade is presumed to regulate tyrosine phosphorylation of various downstream signaling molecules. Expression of Jak-1 and Tyk-2 rescues an IFNα response in certain insensitive cell lines, suggesting that these tyrosine kinases or related members of the Janus family are essential for IFNα action (4, 9).

Several proteins are substrates for IFNα-dependent tyrosine kinase activity. In response to IFNα treatment of cells, the Stat-2, Stat-1α, and Stat-1β components of the transcriptional activator ISGF3α are rapidly phosphorylated on tyrosine and associate with a 48-kDa protein (ISGF3γ) to form an active complex (10–12). This complex translocates to the nucleus and initiates gene transcription during binding to interferon-stimulated response elements (10–13). In addition, the *vav* proto-oncogene product ($p95^{vav}$) is tyrosine-phosphorylated during IFNα stimulation (14); however, its precise role in the signal transduction of IFNα remains to be determined. The involvement of multiple pathways in IFNα signaling is consistent with its pleiotropic biological effects on cells and tissues.

Many growth factor receptors, including those for epidermal growth factor and platelet-derived growth factor, associate directly through their autophosphorylation sites with a common set of signaling proteins that contain SH2 domains, including the phosphatidylinositol (PI) 3′-kinase, Grb-2, SH-PTP2, phospholipase Cγ, and Ras-GAP (15). The receptors for insulin, IGF-1, and IL-4, however, do not strongly associate with most SH2 proteins known to be involved in their signaling pathways (16). Instead, they stimulate tyrosine phosphorylation of docking proteins in the IRS signaling family, notably IRS-1, which binds directly to various SH2 proteins (16–19). We report that IFNα also engages the IRS signaling system, as evidenced by the rapid IFNα-dependent tyrosine phosphorylation of IRS-1 in several hematopoietic cell lines. We also demonstrate that the p85 regulatory subunit of the PI 3′-kinase binds via its SH2 domains the IFNα-induced phosphorylated form of IRS-1, and that PI 3′-kinase activity can be detected in association with IRS-1.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The U-266 (human myeloma), Daudi (lymphoblastoid), and MOLT-4 (acute T-cell leukemia) cell lines were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) or 10% (v/v) defined calf serum (Hyclone Laboratories) and antibiotics. Human recombinant IFNα2 was provided by Hoffmann-La Roche and Schering-Plough. The antiphosphotyrosine monoclonal antibody (4G-10) and the monoclonal antibody against the regulatory subunit of PI 3′-kinase were obtained from Upstate Biotechnology (Lake Placid, NY) and were used for immunoblotting. Nonimmune rabbit IgG was obtained from Sigma. The polyclonal $\alpha IRS\text{-}1^{PH}$ antibody was raised against a GST fusion protein of a region of rat IRS-1 (amino acid residues 1–135) containing its pleckstrin homology domain. An antibody ($\alpha IRS\text{-}1^{CT}$) against a synthetic peptide (TYASINFQKQPEDRQ), corresponding to a sequence present in the C terminus of IRS-1, was raised in rabbits. The polyclonal antibodies against recombinant rat IRS-1 and the p85 regulatory subunit of PI 3′-kinase have been described elsewhwere (20) and were used for immunoprecipitations. A polyclonal antibody against Stat-2 (p113, 186–199) has been described elsewhere (21).

Immunoprecipitations and Immunoblotting—Cells were stimulated with the indicated amounts of IFNα or insulin for the indicated periods of time. After stimulation, the cells were rapidly centrifuged and lysed in a phosphorylation lysis buffer (0.5–1% Triton X-100, 150 mM NaCl, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 50 mM Hepes, 1.5 mM magnesium chloride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). Cell lysates were immunoprecipitated with the indicated antibodies and, after five washes with phosphorylation lysis buffer containing 0.1% Triton X-100, were analyzed by SDS-PAGE and transferred onto nitrocellulose (Schleicher and Schuell) or polyvinylidene difluoride (Immobilon) filters, and the residual binding sites on the

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[1] The abbreviations used are: IFNs, interferons; ISGF3, interferon-stimulated gene factor 3; IRS-1, insulin receptor substrate-1; SH2, Src homology 2; PI 3′-kinase, phosphatidylinositol 3′-kinase; PAGE, polyacrylamide gel electrophoresis; IGF-1, insulin-like growth factor-1; IL, interleukin; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline.

filters were blocked by incubating with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 10% bovine serum albumin) for 1–3 h at room temperature or overnight at 4 °C. The filters were subsequently incubated with the antiphosphotyrosine monoclonal antibody and developed using an enhanced chemiluminesence (ECL) kit following the manufacturer's procedure (Amersham).

Preparation of Glutathione S-Transferase Fusion Proteins—Glutathione *S*-transferase fusion proteins containing the nSH2 and cSH2 domains of PI 3′-kinase (22) were purified from transformed *Escherichia coli* bacteria which were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside as described previously (23). Briefly, after 2 h of additional growth, bacteria were lysed by sonication in phosphate-buffered saline (PBS), pH 7.0. Lysed bacteria were spun for 25 min at 14,000 rpm at 4 °C, and the supernatant was immobilized on glutathione-Sepharose beads (Pharmacia) and used for binding assays.

Assays for PI 3′-Kinase Activity—PI 3′-kinase activity assays on αIRS-1^{PH} immunoprecipitates obtained from IFNα- or insulin-stimulated cells were performed as described previously (24). Briefly, after IFNα or insulin stimulation, serum-starved Daudi cells were washed once with ice-cold PBS and twice with 20 mM Tris, pH 7.5, containing 137 mM NaCl, 1 mM $MgCl_2$, and 100 μM Na_3VO_4 (Buffer A). The cells were lysed in 1 ml of Buffer A containing 1% Nonidet P-40, 10% glycerol, and 0.35 mg/ml phenylmethylsulfonyl fluoride, and insoluble material was removed by centrifugation. Cell lysates were immunoprecipitated with the indicated antibodies, and immunoprecipitates were washed in PBS with 1% Nonidet P-40 and 100 μM Na_3VO_4 (three times), 100 mM Tris, pH 7.5, containing 500 mM $LiCl_2$ and 100 μM Na_3VO_4 (three times), and 10 mM Tris, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4 (twice). The pellets were resuspended in 50 μl of 10 mM Tris, pH 7.5, containing 100 mM NaCl and 1 mM EDTA. 10 μl of 100 mM $MnCl_2$ and 10 μl of phosphatidylinositol (2 μg/μl) sonicated in 10 mM Tris, pH 7.5, 1 mM EGTA were added to each pellet. The reaction was started by the addition of 10 μl of 440 μM ATP containing 30 μCi of [^{32}P]ATP. After incubation for 10 min at 22 °C, the reaction was stopped by the addition of 20 μl of 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate which was coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and visualized and quantitated on a Molecular Dynamics PhosphorImager.

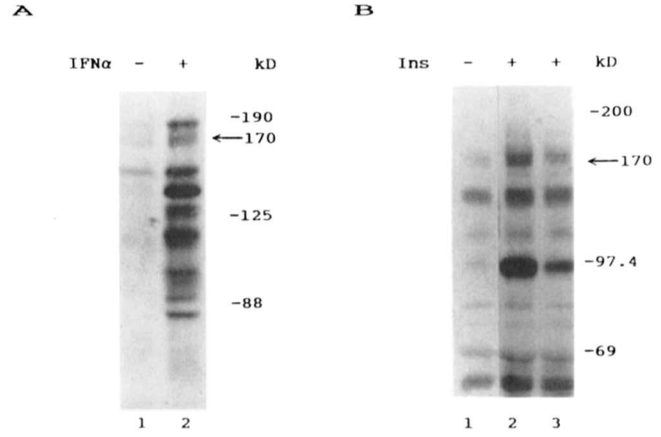

FIG. 1. **Patterns of tyrosine phosphorylation induced by IFNα or insulin in U-266 cells.** Antiphosphotyrosine immunoblots. *A*, cells were incubated in the presence or absence of 10^4 units/ml IFNα for 5 min at 37 °C as indicated. *B*, cells were either not stimulated (*lane 1*) or stimulated with 1 μM insulin for either 5 min (*lane 2*) or 30 min (*lane 3*) at 37 °C as indicated. Equal amounts of protein from total cell lysates (100 μg) were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

We performed experiments in which the patterns of tyrosine phosphorylation induced by IFNα or insulin in hematopoietic cells were studied. We noticed that both IFNα and insulin stimulate tyrosine phosphorylation of a common 170-kDa protein in U-266 cells (Fig. 1). The molecular mass of this protein was similar to the mass of the insulin receptor substrate-1 (17), suggesting that IFNα uses an IRS signaling protein to mediate certain biologic responses.

To determine if IRS-1 is involved in IFNα signaling, lysates from control or stimulated U-266 or Daudi cells were immuno-

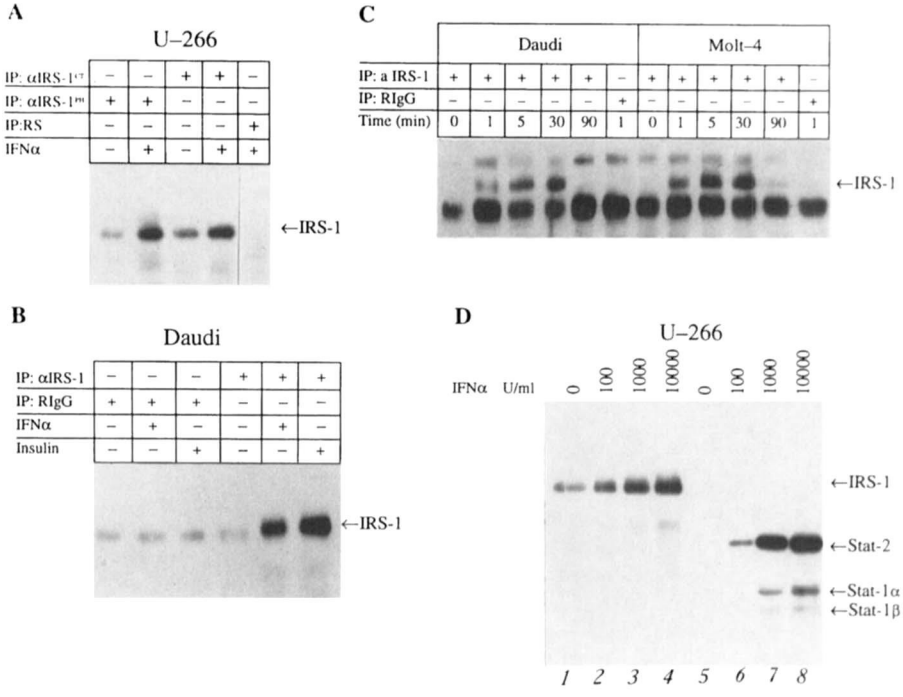

FIG. 2. **Tyrosine phosphorylation of IRS-1 in response to IFNα and insulin.** Antiphosphotyrosine immunoblots. *A*, serum-starved U-266 cells were treated with IFNα (10^4 units/ml) for 5 min at 37 °C as indicated, and cell lysates were immunoprecipitated with αIRS-1^{PH}, αIRS-1^{CT}, or normal rabbit serum (*RS*) as indicated. *B*, Daudi cells were incubated in the presence or absence of IFNα (10^4 units/ml) or insulin (1 μM) for 3 min at 37 °C. Cell lysates were immunoprecipitated with either nonimmune RIgG or a polyclonal antibody against baculovirus-generated rat IRS-1 as indicated. *C*, time course of tyrosine phosphorylation of IRS-1. Cells were stimulated with 10^4 units/ml IFNα for the indicated time points, and cell lysates were immunoprecipitated with either nonimmune RIgG or an antibody against recombinant rat IRS-1 as indicated. *D*, serum-starved U-266 cells were treated for 30 min at 37 °C with the indicated amounts of IFNα, and cell lysates were immunoprecipitated with either αIRS-1^{CT} (*lanes 1–4*) or with a polyclonal antibody against Stat-2 (*lanes 5–8*).

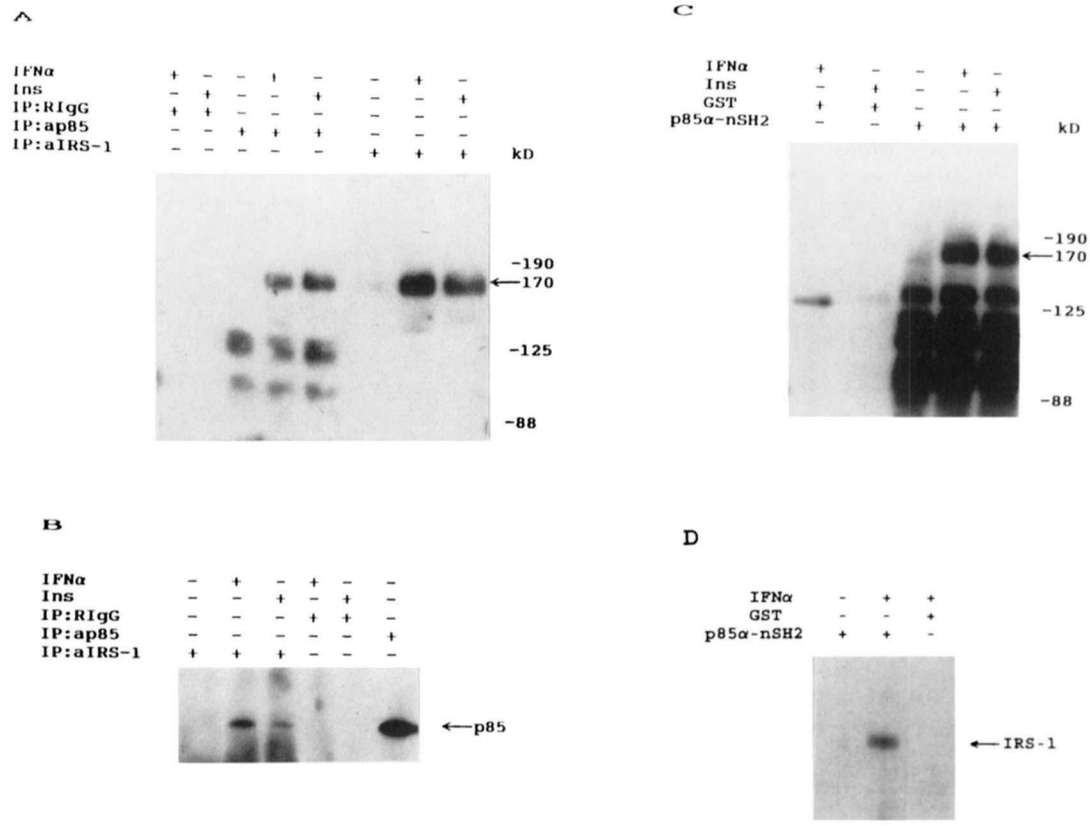

FIG. 3. **Association of IRS-1 with the p85 regulatory subunit of PI 3′-kinase in Daudi cells.** *A*, antiphosphotyrosine immunoblot. Cells (4×10^7/lane) were incubated for 5 min at 37 °C in the presence or absence of IFNα (10^4 units/ml) or insulin (1 μM) as indicated, and cell lysates were immunoprecipitated with normal RIgG, αp85, or αIRS-1^{PH}, as indicated. *B*, cells (5×10^7/lane) were incubated in the presence or absence of IFNα or insulin for 7 min at 37 °C as indicated, and cell lysates were immunoprecipitated with αIRS-1^{PH}, normal RIgG, or αp85 as indicated and immunoblotted with a monoclonal antibody against p85α. *C*, cells (9×10^7/lane) were stimulated with IFNα or insulin for 5 min at 37 °C as indicated, and cell lysates were incubated for 3 h at 4 °C with either GST alone or a GST fusion protein containing the N-terminal SH2 domain of p85α, both of which were bound to glutathione-Sepharose beads. Bound proteins were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine. *D*, cells (1.5×10^8/lane) were incubated for 3 min with IFNα as indicated, and cell lysates were bound to either GST alone or a GST-nSH2 fusion protein. Bound proteins were analyzed by SDS-PAGE and immunoblotted with αIRS-1^{CT}.

precipitated with polyclonal antibodies against IRS-1 and immunoblotted with an antiphosphotyrosine antibody. Basal tyrosine phosphorylation of a 170-kDa protein, corresponding to IRS-1, was consistently detected in U-266 but not in Daudi cells. However, IFNα and insulin strongly stimulated tyrosine phosphorylation of IRS-1 in both cell lines (Fig. 2, *A* and *B*, and data not shown). The IFNα-induced phosphorylation of IRS-1 was rapid and transient, occurring within 1 min of treatment of Daudi or MOLT-4 cells and diminishing after 90 min (Fig. 2*C*). It was also dose-dependent, exhibiting a similar response pattern with the phosphorylation of Stat proteins (Fig. 2*D*).

After tyrosine phosphorylation, IRS-1 binds to several SH2 proteins, including the 85-kDa regulatory subunit (p85) of the PI 3′-kinase (20). To determine whether IRS-1 binds p85 during IFNα stimulation, immunoprecipitates obtained with an anti-p85 antibody from Daudi cell lysates were immunoblotted with antiphosphotyrosine (4G-10). Following IFNα or insulin treatment of the cells, a 170-kDa tyrosine-phosphorylated protein was detected in the anti-p85 immunoprecipitates. This phosphoprotein co-migrated with IRS-1 immunoprecipitated directly with αIRS-1^{PH} (Fig. 3*A*). Moreover, anti-IRS-1 immunoprecipitates contained p85α only after IFNα or insulin stimulation (Fig. 3*B*). The tyrosine-phosphorylated form of IRS-1 from IFNα- or insulin-stimulated cells also bound to a GST fusion protein containing the N-terminal SH2 domain of p85 (Fig. 3*C* and 3*D*). Similar results were obtained using a GST fusion protein containing the C-terminal SH2 domain of p85.[2] Thus, IFNα stimulates the association of IRS-1 with the PI 3′-kinase, and this interaction most likely requires the SH2 domains in p85 (22).

The PI 3′-kinase appears to play an important role in various biological responses and is activated by many growth factors and cytokines (25). Its activation by insulin occurs during association with tyrosine-phosphorylated IRS-1 (20). To determine whether PI 3′-kinase activity is detected in association with IRS-1 during IFNα stimulation, PI 3′-kinase assays were carried out on αIRS-1 immunoprecipitates. Before stimulation, a basal level of PI 3′-kinase activity was detected in the αIRS-1 immunoprecipitates, which was equivalent to the nonspecific activity detected in immunoprecipitates with preimmune rabbit serum (Fig. 4). IFNα or insulin stimulated the association of PI 3′-kinase activity with IRS-1 that was immunoprecipitated specifically with αIRS-1^{PH} (Fig. 4). It is likely that the PI 3′-kinase is activated by IFNα during its association with IRS-1.

Considerable progress has been made in our understanding of IFNα signaling between the plasma membrane and nucleus. The Jak-Stat pathway provides a plausible mechanism for the regulated assembly of ISGF-3, which regulates expression of genes containing the IFN-stimulated response elements (13). The molecular mechanism used by IFNα to regulate other

[2] S. Uddin and L. C. Platanias, unpublished data.

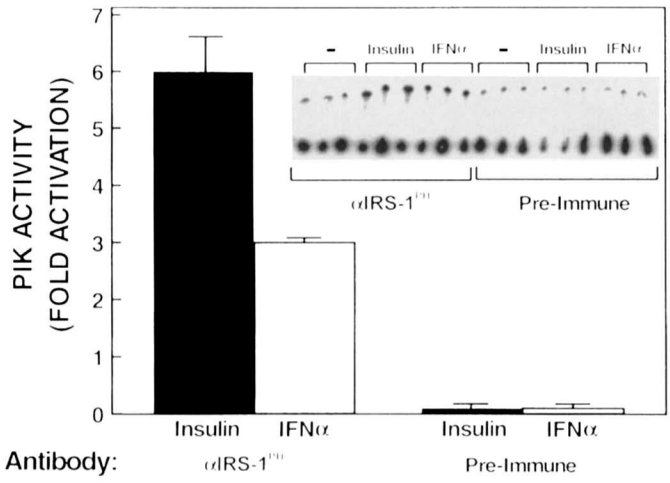

FIG. 4. **Association of PI 3′-kinase activity with IRS-1 during IFNα or insulin stimulation in Daudi cells.** Serum-starved cells were treated for 5 min in the absence or presence of IFNα (10^4 units/ml) or insulin (100 nM) as indicated, cell lysates were immunoprecipitated with αIRS-1^{PH} or preimmune serum, and immunoprecipitates were assayed in triplicate for PI 3′-kinase activity. The *lower spots* in the TLC plate represent the origin, and the *upper spots* represent the phosphorylated phosphatidylinositol substrate. Data were plotted as the average -fold activation ± S.E. for triplicate determinations.

signaling pathways, however, is poorly understood. Our finding that IFNα stimulates tyrosine phosphorylation of IRS-1 suggests the existence of a pathway for the regulated engagement of additional SH2 signaling proteins during IFNα stimulation. It will be important to identify other SH2 proteins engaged by IRS-1 in response to IFNα, as well as in response to IFNβ and IFNω, which also induce its phosphorylation on tyrosine residues.[2]

IRS-1 plays a central role in the signal transduction of insulin and interleukin-4, and its signaling functions appear to be essential for the mitogenic effects of these ligands (19). Recent evidence has suggested that IRS-1 may also be involved in growth hormone signaling (26). The protein contains many potential tyrosine phosphorylation sites in various hydrophobic contexts (16). These tyrosine residues play a dual role as substrates for upstream tyrosine kinases and as specific docking sites for downstream SH2 proteins. At least eight tyrosine residues in IRS-1 undergo phosphorylation by the activated insulin receptor, including residues 460, 608, 628, 939, and 987, which are in YXXM/YMXM motifs and bind to p85 which activates the PI 3′-kinase (20). Three other motifs are also phosphorylated by the insulin receptor, including Y^{895}VNI, which binds Grb-2, and Y^{1172}IDL and Y^{1222}ASI which bind SH-PTP2 (27). Studies to determine the motifs of IRS-1 that are tyrosine-phosphorylated in response to IFNα should provide valuable information on the mechanisms by which the signaling specificity is established through the common use of this protein by different cytokines and growth factors.

We currently think that the insulin receptor tyrosine kinase regulates the insulin-dependent phosphorylation of IRS proteins, whereas the receptors for IL-4 and IFNα use Janus family tyrosine kinases to accomplish such phosphorylation; however, direct evidence that IRS-1 acts as a substrate for Janus kinases during IFNα stimulation remains to be obtained. Many receptors activate the Janus family of tyrosine kinases, but most of them do not phosphorylate IRS-1. The identity of the elements responsible for this selectivity are unknown. Interestingly, the receptors for insulin, IGF-1, and IL-4 contain a common amino acid sequence motif, LxxxxN-PxYxxss, which appears to contribute to the interaction of these receptors with IRS-1 (28); however, this sequence motif is not found in the cloned components of the Type I IFN receptor (8, 29), suggesting that a different motif may be involved or another subunit remains to be found.

The shared use of the IRS signaling pathway establishes a common link between apparently distinct signaling systems for insulin, IGF-1, IL-4, and IFNα. Recently, Larner *et al.* (30) suggested that IL-4 attenuates the transcriptional activation of IFN-induced cellular gene expression in monocytes and related cell lines. Although the mechanism of this phenomenon is unknown, our results raise the possibility of an antagonism between IFNα and IL-4 occurring through the common use of proteins in the IRS signaling system. If this hypothesis is correct, then insulin and IGF-1 may also influence IFNα signaling. Additional work in this area should reveal important relationships between these apparently distinct factors and others as well.

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