THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 1, Issue of January 5, pp. 278–282, 1996
Printed in U.S.A.

The Type I Interferon Receptor Mediates Tyrosine Phosphorylation of Insulin Receptor Substrate 2*

(Received for publication, October 27, 1995)

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Binding of interferon α (IFNα) to its receptor induces activation of the Tyk-2 and Jak-1 tyrosine kinases and tyrosine phosphorylation of multiple downstream signaling elements, including the Stat components of the interferon-stimulated gene factor 3 (ISGF-3). IFNα also induces tyrosine phosphorylation of IRS-1, the principle substrate of the insulin receptor. In this study we demonstrate that various Type I IFNs rapidly stimulate tyrosine phosphorylation of IRS-2. This is significant since IRS-2 is the major IRS protein found in hematopoietic cells. The IFNα-induced phosphorylated form of IRS-2 associates with the p85 regulatory subunit of the phosphatidylinositol 3′-kinase, suggesting that this kinase participates in an IFNα-signaling cascade downstream of IRS-2. We also provide evidence for an interaction of IRS-2 with Tyk-2, suggesting that Tyk-2 is the kinase that phosphorylates this protein during IFNα stimulation. A conserved region in the pleckstrin homology domain of IRS-2 may be required for the interaction of IRS-2 with Tyk-2, as shown by the selective binding of glutathione *S*-transferase (GST) fusion proteins containing the IRS-2-IH1^{PH} or IRS-1-IH1^{PH} domains to Tyk-2 but not other Janus kinases *in vitro*.

Type I interferons (IFNs)[1] are pleiotropic cytokines that exhibit multiple biological effects on normal and malignant cells, including antiproliferative, antiviral, and immunomodulatory activities (1). Although the precise mechanisms by which Type I IFNs exhibit their biological effects remain unknown, significant advances have been made recently on our understanding of the early events of signaling by the Type I IFN receptor (IFNR). Two kinases of the Janus family, Tyk-2 and Jak-1, are associated with components of the the Type I IFNR complex (2, 3). IFNα stimulates tyrosine phosphorylation of the α and β subunits of the Type I IFNR (4–7) and activation of Tyk-2 and Jak-1 (2, 8–10). During IFNα stimulation, the Stat-2, Stat-1α, and Stat-1β components of the transcriptional activator ISGF3α are rapidly phosphorylated on tyrosine and associate with ISGF3γ to form an active complex (11–14). This complex translocates to the nucleus to initiate transcription of interferon-stimulated genes during binding to interferon-stimulated response elements (11–14). Tyk-2 and Jak-1 are essential for this response, as IFNα-insensitive cells are rescued by expression of Tyk-2 or Jak-1 (8, 15).

Although the molecular link between the Type I IFN receptor and ISGF3α provides an important paradigm for interferon signaling, other signaling pathways may also be involved. To our surprise, IFNα stimulates tyrosine phosphorylation of IRS-1 (16). This is somewhat counterintuitive, since IRS-1 clearly mediates cell growth and metabolism during insulin/IGF-1 and IL-4 stimulation (17). Moreover, IRS-1 appears to mediate SV40 large T antigen transformation (18). The IRS-1 signaling function is best characterized during insulin stimulation, as it is phosphorylated at multiple tyrosine residues which bind to the SH2 domains in PI 3′-kinase regulatory subunits (p85α/p85β/p55^{PIK}), Grb-2, Nck, Fyn,[2] and SH-PTP2 (19–22). IRS-1 associates with other proteins, including integrins ($\alpha_v\beta_3$) (23) and 14-3-3[3] proteins, and such associations may play an important role in the biological action of these proteins. In addition to IL-4 receptors, a growing number of cytokine receptors mediate tyrosine phosphorylation of IRS-1, including IL-9, IL-13, growth hormone, and leukemia inhibitory factor (18, 24–29). As a consequence of these and other interactions, IRS-1 mediates multiple downstream signals, including the direct activation of PI 3′-kinase and SH-PTP2, the indirect stimulation of mitogen-activated protein kinase and p70^{S6K}, and other events which regulate gene expression and stimulate mitogenesis and glucose transport (18, 23, 30–34).

Interest in a putative "IRS-2" increased recently when it was discovered that IRS-1$^{(-/-)}$ mice survive, reproduce, and display only mild insulin resistance (35, 36). The mice are small during their life, but display no other obvious phenotypic changes, including an apparently normal immune response.[4] Many hematopoietic cells do not contain IRS-1, but express the newly cloned isoform, IRS-2 (37). In this study, we show that Type I IFNs stimulate tyrosine phosphorylation of IRS-2 in several cell lines of diverse hematopoietic origin, and that, after tyrosine phosphorylation, IRS-2 associates with the p85 regulatory subunit of the PI 3′-kinase. Furthermore, we demonstrate that

* This work was supported in part by grants from the Department of Veterans Affairs and the Hairy Cell Leukemia Foundation (to L. C. P.) and by National Institutes of Health Grants DK43808 and DK38712 (to M. F. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

[2] X.-J. Sun, S. Pons, T. Asano, M. G. Myers, E. Glasheen, and M. F. White, submitted for publication.

[3] T. Asano and M. F. White, unpublished data.

[4] C. R. Kahn, personal communication.

the pleckstrin homology (PH) domains of IRS-2 and IRS-1 associate with the tyrosine kinase Tyk-2 *in vitro*, suggesting that this motif in IRS-proteins mediates their interaction with the Type I IFN receptor complex.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The U-266 (human multiple myeloma), KG1 (human acute myeloid leukemia), KG1A (human acute myeloid leukemia), HEL (human acute erythroleukemia), and Molt-4 (acute T-cell lymphocytic 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, Logan, UT) and antibiotics. The IL-3-dependent mouse myeloid FDCP-2 cell line was grown in RPMI-10% fetal bovine serum with the addition of 5% WEHI supernatant as a source for IL-3. Human recombinant IFNα2 was provided by Dr. Michael Brunda (Hoffmann-La Roche). Human recombinant IFNβ-1b (IFNβ) was provided by Dr. Gary Williams (Berlex Laboratories, Richmond, CA). Human recombinant IFNω was a gift (to Dr. M. O. Diaz) from Dr. G. Addolf (Ernst Boehringer Institute fur Arzneimittelforschung, Vienna, Austria). Human recombinant IFNγ was provided by Genentech Inc. (South San Francisco, CA). Mouse interferon α/β was purchased from Lee and Biomolecular Research Laboratories (San Diego, CA). The antiphosphotyrosine monoclonal antibody (4G-10) and a monoclonal antibody against the p85 regulatory subunit of PI 3′-kinase were obtained from Upstate Biotechnology (Lake Placid, NY) and were used for immunoblotting. The polyclonal αIRS-1^{CT}, αIRS-2, and αp85 polyclonal antibodies have been described previously (16, 37, 38). The polyclonal anti-Tyk-2 antibody has been raised against a synthetic peptide corresponding to the COOH-terminal 15 amino acids of Tyk-2 (5, 39). The monoclonal antibody against Tyk-2 was obtained from Transduction Laboratories (Lexington, KY) and was used for immunoblotting. Polyclonal antibodies against Jak-2 were obtained from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody against Jak-1 was obtained from Upstate Biotechnology.

Immunoprecipitations and Immunoblotting—Cells were stimulated with the indicated amounts of different interferons or insulin (1 μM) for the indicated time periods. In some experiments, the cells were serum-starved for 1–5 h in serum-free RPMI 1640 or Dulbecco's modified Eagle's medium (Life Technologies, Inc.) prior to stimulation. After stimulation, the cells were lysed in a phosphorylation lysis buffer (0.5% 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) for 60 min at 4 °C. Insoluble material was removed by centrifugation, and cell lysates were immunoprecipitated with the indicated antibodies using protein G-Sepharose (Pharmacia Biotech Inc.). After five washes with phosphorylation lysis buffer containing 0.1% Triton X-100 or without Triton X-100 in some experiments, proteins were analyzed by SDS-PAGE and transferred onto polyvinylidene difluoride filters (Immobilon, Millipore). The residual binding sites on the filters were blocked by incubating with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20), 10–20% 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 recommended procedure (Amersham).

Preparation of Glutathione S-Transferase Fusion Proteins and Binding Studies—The pGEX constructs encoding the IH1^{PH} domains of IRS-1 and IRS-2 have been described elsewhere.[5] Preparation of glutathione *S*-transferase fusion proteins for binding experiments with cell lysates from IFNα-stimulated cells was performed as described previously (16, 40).

RESULTS AND DISCUSSION

We investigated the tyrosine phosphorylation of IRS-2 during IFNα stimulation of various human hematopoietic cells. After immunoprecipitation using specific anti-IRS-1 or anti-IRS-2 antibodies, proteins were analyzed by SDS-PAGE and immunoblotted with an antiphosphotyrosine monoclonal antibody. As shown previously (14), IFNα stimulated tyrosine phosphorylation of IRS-1 in U-266 myeloma cells; it also stimulated the tyrosine phosphorylation of IRS-2 (Fig. 1). The protein migrated slightly above IRS-1, as predicted by its slightly larger size (37). Moreover, IFNα stimulated the phosphorylation of IRS-2 in the KG1 and KG1A acute myeloid leukemia cell lines (Fig. 1), which have been shown previously to respond to the antiproliferative effect of IFNα (41).

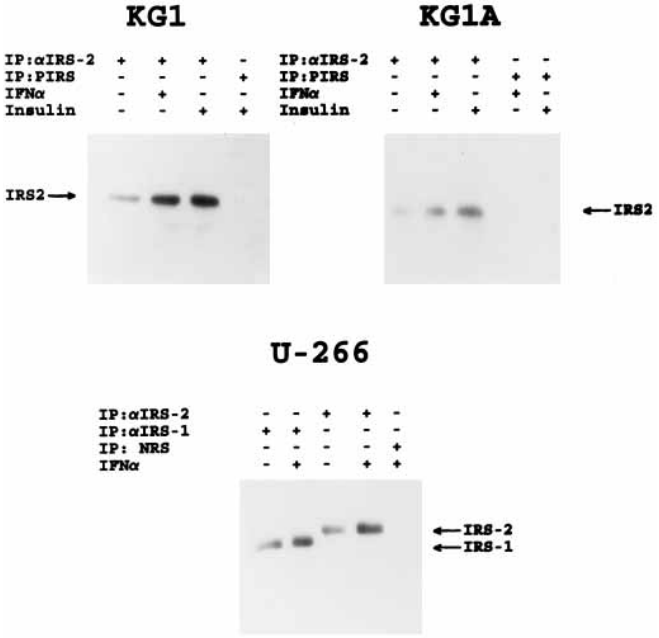

FIG. 1. **IFNα induces tyrosine phosphorylation of IRS-2.** Antiphosphotyrosine imunoblots are shown. *Upper panel*, serum-starved KG1 (4.8×10^7/lane) or KG1A (2.8×10^7/lane) cells were incubated for 5 min at 37 °C in the presence or absence of IFNα (10^4 units/ml) or insulin (1 μM) as indicated, and cell lysates were immunoprecipitated with αIRS-2 or preimmune rabbit serum (*PIRS*) as indicated. *Lower panel*, serum-starved U-266 cells (1.4×10^7/lane) were incubated for 5 min at 37 °C in the presence or absence of IFNα (10^4 units/ml) as indicated, and cell lysates were immunoprecipitated with αIRS-1^{CT}, αIRS-2, or normal rabbit serum (*NRS*) as indicated.

The kinetics of IRS-2 phosphorylation was examined in HEL and FDCP-2 cells. Treatment of HEL cells with human IFNα, or FDCP-2 cells with mouse IFNα/β, stimulated maximum tyrosine phosphorylation of IRS-2 within 5 min (Fig. 2, *A* and *B*). The phosphorylation gradually declined and became undetectable after 90 min, suggesting that the phosphorylation of IRS-2 is rapid and transient during IFNα stimulation. Similar time courses for the phosphorylation of IRS-1 have been observed previously during IFNα or insulin stimulation in other cell types. The mechanism of dephosphorylation is unknown and may involve degradation of IRS-2, activation of a phosphatase, or down-regulation of the receptor.

The functional Type I IFN receptor complex is composed of several components, including the Tyk-2 and Jak-1 kinases (2, 3), which are responsible for signal transmission. The time course of IRS-2 phosphorylation was studied in parallel with the phosphorylation of Tyk-2 in U-266 cells. Tyk-2 migrates as a 135-kDa protein during SDS-PAGE. The IFNα-induced phosphorylation of Tyk-2 exhibited a similar time course with the phosphorylation of IRS-2. Interestingly, a 135-kDa tyrosine-phosphorylated protein that co-migrated with Tyk-2 was co-immunoprecipitated by the αIRS-2 antibody in some experiments, suggesting that Tyk-2 associates with IRS-2 (Fig. 2*C*). However, this association was not observed consistently, possibly due to low stoichiometry of such interactions; similar variable results were reported previously for the association between insulin receptors and IRS-1 (42). Apparently, the as-

[5] L. Yenush, K. Makati, J. Smith-Hall, O. Ishibashi, M. G. Myers, Jr., and M. F. White, submitted for publication.

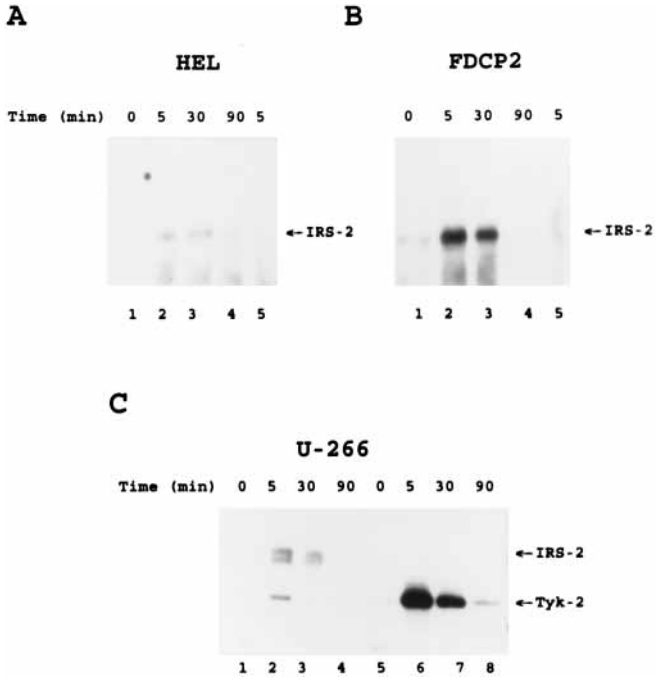

FIG. 2. **Kinetics of IFNα-dependent phosphorylation of IRS-2.** Serum-starved HEL cells (2.8×10^7/lane) (*A*) or FDCP-2 cells (2.5×10^7/lane) (*B*) were treated with human IFNα or mouse IFNα/β, respectively, for the indicated times at 37 °C. Cell lysates were immunoprecipitated with αIRS-2 (*lanes 1–4*) or normal rabbit serum (*lane 5*) and immunoblotted with antiphosphotyrosine. *C*, serum-starved U-266 cells (2.1×10^7/lane) were treated with IFNα for the indicated times. Cell lysates were immunoprecipitated with either αIRS-2 (*lanes 1–4*) or αTyk-2 (*lanes 5–8*) as indicated and immunoblotted with antiphosphotyrosine.

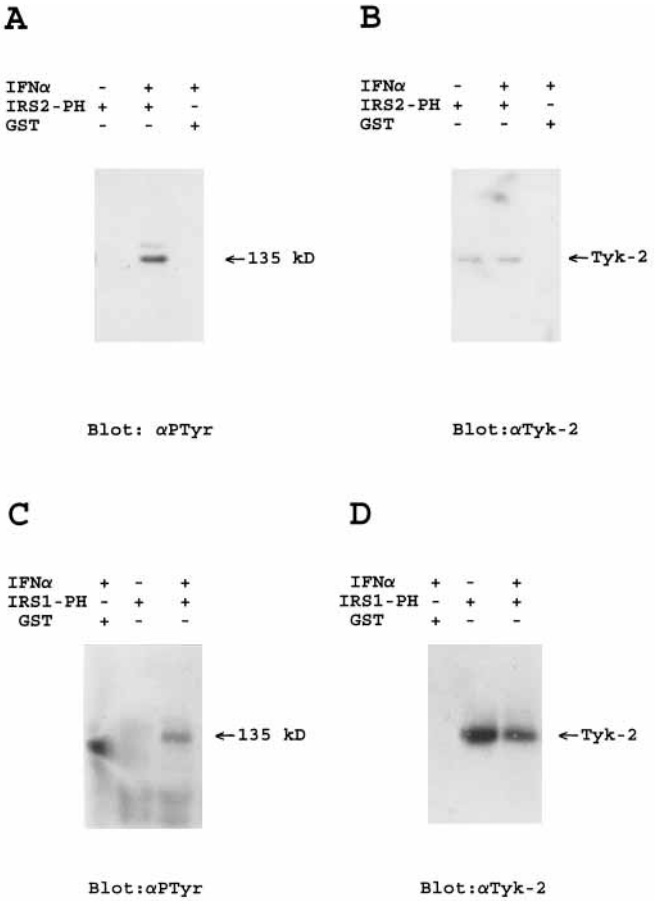

FIG. 3. **Association of the pleckstrin homology domains of IRS proteins with Tyk-2.** *A*, U-266 cells (2.1×10^7/lane) were treated with 10^4 units/ml IFNα for 5 min at 37 °C as indicated, and cell lysates were bound to either IRS-2/PH-GST or GST alone as indicated. Proteins were analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. *B*, the blot shown in *A* was stripped and reblotted with a monoclonal antibody against Tyk-2. *C*, serum-starved Daudi cells (5.8×10^7/lane) were treated with 10^4 units/ml IFNα for 2 min at 37 °C as indicated, and cell lysates were bound to either IRS-1/PH-GST or GST alone. Proteins were analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. *D*, the blot shown in *C* was stripped and reblotted with a monoclonal antibody against Tyk-2.

sociations between receptor complexes and IRS proteins are weak and/or transient, but sufficient to mediate specific phosphorylation.

The mechanism of specific recognition of IRS proteins by the Type I IFN receptor complex is unknown. Two regions conserved in the NH_2 terminus of IRS-1 and IRS-2, called the $IH1^{PH}$ domain and $IH2^{PTB}$ domains, mediate the association with the insulin receptor (37, 43). The $IH1^{PH}$ domain resembles a pleckstrin homology domain (PH), whereas the $IH2^{PTB}$ domain is a phosphotyrosine binding domain (37). The $IH1^{PH}$ domain is important for the sensitive interaction between the insulin receptor and IRS-1, although the mechanism is unknown (43), while the $IH2^{PTB}$ domain binds to phosphorylated L*XXXX*NP*XY*S*X*S motifs in the insulin and IL-4 receptors (44); however, the known components of the Type I IFN receptor do not contain such motifs (3, 45, 46). To determine whether the $IH1^{PH}$ motif of IRS-2 mediates the interaction with the Type I IFN receptor complex, a GST fusion protein containing the $IH1^{PH}$ domain of IRS-2 was incubated with lysates from human cells. Tyk-2 associated with the IRS-2/$IH1^{PH}$ domain, suggesting that this motif may provide a molecular link to the Type I IFN receptor complex (Fig. 3, *A* and *B*). Similarly, a GST fusion protein containing the $IH1^{PH}$ domain of IRS-1 (IRS-1/$IH1^{PH}$) also bound to Tyk-2 (Fig. 3, *C* and *D*). In contrast, the GST-IRS-1/$IH1^{PH}$ and GST-IRS-2/$IH1^{PH}$ fusion proteins did not bind to Jak-1 present in extracts from IFNα-stimulated cells (data not shown), suggesting that this motif in IRS proteins interacts specifically with Tyk-2 but not Jak-1. Additional studies using various mutations in the $IH1^{PH}$ domains will be needed to establish the significance of this result in the biological context.

In addition to IFNα, IFNβ, and IFNω bind to the Type I IFN receptor and stimulate tyrosine phosphorylation of Tyk-2, Jak-1, and Stats (5). When the tyrosine phosphorylation of IRS-2 in response to IFNβ and IFNω was studied, we found that both IFNs induce tyrosine phosphorylation of IRS-2 (Fig. 4*A*). Similarly, IFNβ and IFNω stimulated tyrosine phosphorylation of IRS-1 in Molt-4 cells (Fig. 4*B* and data not shown). In contrast, IFNγ binds to the Type II IFN receptor, which utilizes the Jak-1 and Jak-2 tyrosine kinases for signal transduction (8–10, 14, 47). IFNγ did not stimulate tyrosine phosphorylation of IRS-2 or IRS-1 in U-266 or Molt-4 cells, despite the fact that Jak-2 is expressed and tyrosine-phosphorylated by IFNγ in both cell lines (Fig. 4*C* and data not shown). Consistent with this finding, binding studies with the GST-IRS-2-$IH1^{PH}$ and GST-IRS-2-$IH1^{PH}$ fusion proteins demonstrated that these motifs do not bind to the IFNγ-regulated tyrosine kinase Jak-2 *in vitro* (Fig. 4, *D* and *E*, and data not shown). Taken altogether, these findings suggest that Type I IFNs utilize IRS proteins during signal transmission, and that IRS proteins represent a point of diversity between the Type I and Type II IFN receptors in hematopoietic cells.

We have previously established that the p85 regulatory subunit of the PI 3′-kinase associates with tyrosine-phosphorylated IRS-1 in an IFNα-dependent manner (16). To determine whether IRS-2 associates with p85 during IFNα stimulation,

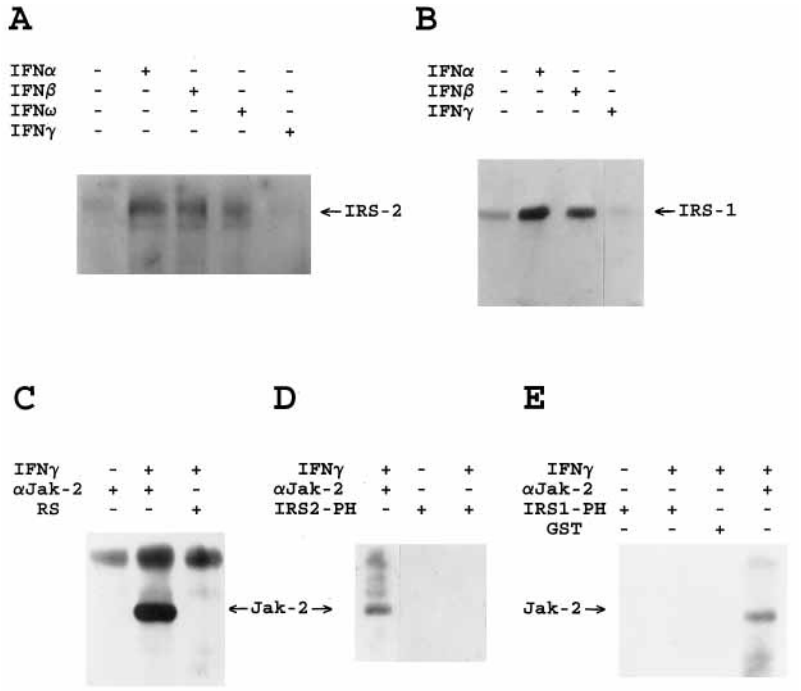

FIG. 4. **Tyrosine phosphorylation of IRS proteins by IFNβ and IFNω but not IFNγ.** Antiphosphotyrosine immunoblots are shown. *A*, serum-starved U-266 cells were treated with 2×10^4 units/ml of the indicated IFNs for 10 min at 37 °C, and cell lysates were immunoprecipitated with αIRS-2. *B*, serum starved Molt-4 cells were treated with 2×10^4 units/ml of the indicated interferons for 5 min at 37 °C, and cell lysates were immunoprecipitated with αIRS-1^{CT}. *C*, U-266 cells were treated with 10^4 units/ml IFNγ for 5 min at 37 °C, and cell lysates were immunoprecipitated with an αJak-2 polyclonal antibody or normal rabbit serum (*RS*) as indicated. *D*, U-266 cells were treated with 10^4 units/ml IFNγ for 5 min at 37 °C as indicated. Cell lysates were either immunoprecipitated with an αJak-2 antibody or were bound to GST-IRS2-PH as indicated prior to SDS-PAGE analysis and immunoblotting with antiphosphotyrosine. *E*, U-266 cells were treated with 10^4 units/ml IFNγ for 5 min at 37 °C as indicated. Cell lysates were either immunoprecipitated with an αJak-2 antibody or were bound to GST-IRS1-PH as indicated prior to SDS-PAGE analysis and immunoblotting with antiphosphotyrosine.

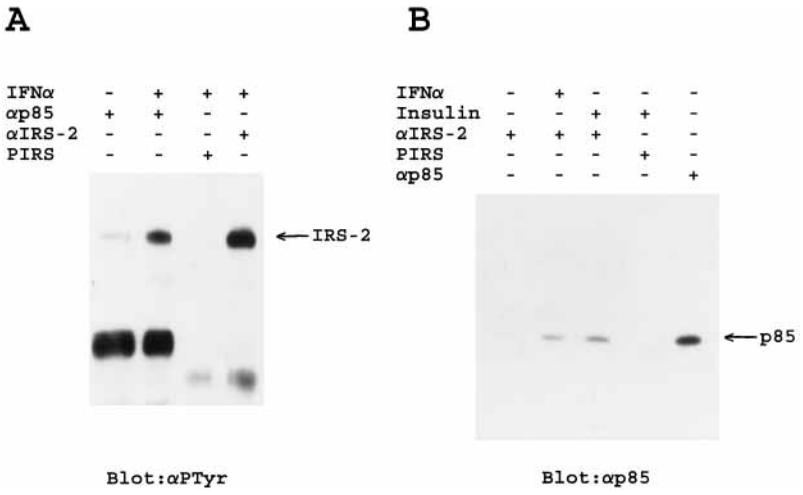

FIG. 5. **IFNα-dependent association of IRS-2 with the p85 regulatory subunit of the PI 3′-kinase.** *A*, serum-starved KG1 cells were incubated for 5 min at 37 °C in the presence or absence of IFNα as indicated, and cell lysates were immunoprecipitated with the indicated antibodies and immunoblotted with antiphosphotyrosine. *B*, serum-starved KG1 cells were treated for 5 min with IFNα or insulin as indicated, and cell lysates were immunoprecipitated with the indicated antibodies and immunoblotted with a monoclonal antibody against p85α.

lysates from IFNα-stimulated cells were immunoprecipitated with an αp85 polyclonal antibody and immunoblotted with antiphosphotyrosine. A 180-kDa phosphoprotein was clearly detectable in the αp85 immunoprecipitates from IFNα-stimulated cells and co-migrated with IRS-2 that was immunoprecipitated directly with the αIRS-2 antibody (Fig. 5*A*). In experiments in which cell lysates were immunoprecipitated with αIRS-2 and immunoblotted with a monoclonal antibody against p85α, we noticed that p85α associates with IRS-2 in an IFNα-dependent manner (Fig. 5*B*). Thus, IRS-2, in addition to IRS-1, provides a link between the Type I IFN receptor and the PI 3′-kinase in hematopoietic cells.

Originally, IRS-1 was thought to be a specific substrate for the insulin and IGF-1 receptors; however, a more complicated picture emerged with the appreciation that other receptors engage and phosphorylate IRS-1 (16, 17, 24–29). The purification and cloning of IRS-2 further complicates the matter as the tyrosine phosphorylation motifs are not the same (37). Insulin, IL-4, and IFNα stimulate tyrosine phosphorylation of IRS proteins in hematopoietic cells; however, the biological effects of these factors are not identical. Insulin/IGF-1 and IL-4 stimulate DNA synthesis and growth of hematopoietic cells, and this response requires IRS proteins (17, 37), while IFNα inhibits cell proliferation (1, 48). Thus, phosphorylation of IRS proteins may not always mediate the same signals. For example, expression of IL-4 receptors and the γ-chain of the IL-2 receptor in L6 myoblasts mediates IRS-1 phosphorylation, but IL-4 does not activate p21^{ras} or stimulate glucose uptake in this cell

background (49, 50).

The PI 3′-kinase is activated during association with IRS-1 in response to insulin stimulation. Although monophosphopeptides mimic the ability of phosphorylated IRS-1 to activate the PI 3′-kinase, activation by a biphosphopeptide (based on the amino acid sequence around Tyr^{608} and Tyr^{628} in IRS-1) is more sensitive and reflects more closely the activation by recombinant phosphorylated IRS-1 (38, 51). The activity of the PI 3′-kinase during association with IRS-1 may depend on the pattern of tyrosine phosphorylation of the nine Y*XX*M motifs. The sites of IRS-1 and IRS-2 tyrosine-phosphorylated during IFNα stimulation have not been determined. Identification of such sites should provide valuable information on the mechanisms of regulation of PI 3′-kinase activity during IFNα stimulation and possibly provide an explanation for the distinct characteristics of the ultimate biological responses between IFNs and insulin.

The phosphorylation of IRS proteins is a common event in the signaling pathways of the Type I IFNs. In contrast, IFNγ does not stimulate phosphorylation of IRS proteins in U-266 or Molt-4 cells; however, IFNγ stimulates tyrosine phosphorylation of IRS-1 in 3T3-F442A adipocytes, which correlates with tyrosine phosphorylation of Jak-2 (26). This contradicts our results in the Molt-4 and U-266 hematopoietic cell lines studied here. Although Jak-2 is clearly tyrosine-phosphorylated by IFNγ in both cell lines, it is possible that activation of Jak-2 alone is not sufficient for tyrosine phosphorylation of IRS proteins, as we failed to observe phosphorylation of IRS-1 or IRS-2. Tyk-2 but not Jak-2 associates with the pleckstrin homology domains of IRS-1 and IRS-2 ($IH1^{PH}$) which may mediate, at least partially, the recognition of the substrate by the activated Type I IFN receptor in hematopoietic cells. It is possible that the recognition of IRS-1 by the activated Type II IFN receptor in 3T3-F442A cells requires an unknown signaling protein, which is not present in hematopoietic cells.

The biological consequences of tyrosine phosphorylation of IRS proteins by Type I IFNs remain unknown. The specific signals required for the expression of the antiproliferative effects of IFNs have not been identified. There is no evidence available that the Stat pathway mediates this effect. However, it is difficult to understand how IRS proteins are involved in the generation of antiproliferative signals. It is possible that differential phosphorylation of IRS proteins leads to the formation of active *versus* inactive or entirely unique signaling complexes. Studies to determine whether IRS-1 and/or IRS-2 expression in resistant cells restores IFNα sensitivity should provide interesting information on the mechanisms of regulation of cell growth by the Type I IFN receptor, as well as the specific functions mediated by each of these proteins.

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