Activation of the Phosphatidylinositol 3-Kinase Serine Kinase by IFN-α[1]

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During engagement of the type I IFN receptor, IRS-1 is phosphorylated on tyrosine and associates with the p85 regulatory subunit of the phosphatidylinositol (PI) 3′-kinase, which is a dual-specificity enzyme possessing both lipid and serine kinase activities. We sought to determine whether treatment of cells with IFN-α activates the PI 3′-kinase serine kinase. ^{32}P-labeling experiments and phosphoaminoacid analysis of immunoprecipitated IRS-1 protein demonstrated that, in addition to tyrosine phosphorylation, IFN-α induces its phosphorylation on serine residues. In vitro kinase assays on αIRS-1 immunoprecipitates also demonstrated IFN-α-dependent serine phosphorylation of IRS-1, suggesting that the protein associates with an IFN-α-regulated serine kinase. Furthermore, IFN-α-dependent phosphorylation of IRS-1 was detected in in vitro kinase assays on αp85 immunoprecipitates, and was inhibited by pretreatment of cells with the specific PI 3′-kinase inhibitor wortmannin, consistent with a regulatory role of the PI 3′-kinase serine kinase on the phosphorylation of the protein. Treatment of cells with wortmannin also inhibited the phosphorylation of the p85 subunit of PI 3′-kinase and the type I IFN-regulated activation of the Map kinase, but had no inhibitory effect on the IFN-α-induced activation of Tyk-2 and Jak-1 kinases nor on the activation of Stat-1, Stat-2, and Stat-3. Taken all together, these data establish that the PI 3′-kinase serine kinase is activated by IFN-α and may play an important role in the transmission of type I IFN receptor-generated signals. ***The Journal of Immunology,* 1997, 158: 2390–2397.**

Several of the early steps in signaling by the type I IFN receptor have been identified. IFN-α treatment of human cells induces tyrosine phosphorylation of the α (1–4) and β (2) subunits of the type I IFN receptor, and activation of the receptor-associated Tyk-2 and Jak-1 kinases (5–7). Activation of these tyrosine kinases appears to regulate the phosphorylation of various downstream signaling elements, including Stat-1 (8–10), Stat-2 (8–10), Stat-3 (11), and Vav (12). In addition, IFN-α activates the insulin receptor substrate (IRS)[3] signaling system, as evidenced by the IFN-α-dependent tyrosine phosphorylation of IRS-1 and IRS-2, and their association with the SH2-containing p85 regulatory subunit of the PI 3′-kinase (13, 14).

Although the importance of tyrosine kinases in IFN-α signaling is well established, the role that serine kinases play in the generation of IFN-α signals is not well defined. In a previous study, we demonstrated that during IFN-α stimulation PI 3′-kinase activity is detected in association with IRS-1, suggesting that the lipid kinase activity of the p110 subunit of PI 3′-kinase is activated by IFN-α (13). It is now well established that in addition to lipid kinase activity, the PI 3′-kinase exhibits serine kinase activity (15, 16), which is activated during insulin stimulation (17, 18).

In the present study we sought to determine if IFN-α activates the serine kinase activity of the PI 3′-kinase. Our data show that treatment of cells with IFN-α stimulates the PI 3′-kinase serine kinase. A major substrate for its activity appears to be IRS-1, as evidenced by the detection of IFN-α-dependent serine phosphorylation of IRS-1 and the inhibition of such phosphorylation by the PI 3′-kinase inhibitor, wortmannin. Furthermore, the PI 3′-kinase serine kinase may regulate engagement of the Map kinase in type I IFN signaling, as suggested by the inhibition of type I IFN-induced activation of the Map kinase by wortmannin.

Materials and Methods

Cells and reagents

The U-266 (multiple myeloma), Daudi (lymphoblastoid), and HSB-2 (acute T cell lymphocytic leukemia) human cell lines were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) FBS (Life Technologies, Inc.) or 10% (v/v) defined calf serum (HyClone Laboratories, Logan, UT) and antibiotics. Human recombinant IFN-α2 was provided by Hoffmann-La Roche (Nutley, NJ). Human recombinant IFN-β was from Biogen Incorporation (Cambridge, MA). The antiphosphotyrosine mAb (4G-10) and the mAb against the p85α regulatory subunit of PI 3′-kinase were obtained from Upstate Biotechnology (Lake Placid, NY), and were used for immunoblotting. The polyclonal Abs against the p85 regulatory subunit of PI 3′-kinase and Jak-1 were also from Upstate Biotechnology and were used for immunoprecipitations. The polyclonal αIRS-1^{CT} Ab, the Abs against the α and β_S subunits of the type I IFN receptor, and the Ab against the tyrosine kinase Tyk-2 have been previously described (2, 13, 19, 20). The polyclonal Ab against the activated/phosphorylated form of Map kinase was obtained from New England

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[3] Abbreviations used in this paper: IRS, insulin receptor substrate; PI, phosphatidylinositol; EMSA, electrophoretic mobility shift assay; ISRE, IFN-stimulated response elements; SIE, Sis-inducible element.

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Biolabs. A mAb against Erk-2 was obtained from Transduction Laboratories (Lexington, KY).

Immunoprecipitations and immunoblotting

Cells were stimulated with IFN-α (1–2 × 10^4 U/ml) 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 PMSF, and 10 μg/ml aprotinin). Cell lysates were immunoprecipitated with the indicated Abs and, after five washes with phosphorylation lysis buffer containing 0.1% Triton X-100, were analyzed by SDS-PAGE and transferred on polyvinylidene-dilfluoride (Immobilon, Millipore, Bedford, MA) filters, and 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) with 10 to 20% BSA for 1 to 3 h at room temperature or overnight at 4°C. The filters were subsequently incubated with the indicated Abs and developed using an enhanced chemiluminescence kit following the manufacturer's procedure (Amersham, Arlington Heights, IL).

In vitro kinase assays and phosphoaminoacid analysis

U-266 cells were serum starved by incubation in serum-free DMEM medium at 37°C for 2 h. The cells were subsequently incubated in the presence or absence of 1 μM insulin or 10^4 U/ml of IFN-α for the indicated times. After treatment, the cells were lysed in phosphorylation lysis buffer, and cell lysates were immunoprecipitated with the indicated Abs. In vitro kinase assays were performed as previously described (21), or using a modified procedure as follows: immunoprecipitated proteins on protein G-sepharose beads were washed three times with phosphorylation lysis buffer containing 0.1% Triton X-100 and two times with in vitro kinase buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4). The immunocomplex-protein G sepharose beads were resuspended in 50 μl of in vitro kinase buffer containing 10 mM $MnCl_2$ and 10 to 20 μCi of [γ-^{32}P]ATP. The beads were incubated for 20 min at room temperature, and the reaction was terminated by adding 1 ml of ice-cold buffer A (0.1% Nonidet P-40, 20 mM EDTA, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4). The immunoprecipitates were washed once with buffer A and proteins were analyzed by SDS-PAGE. The proteins were subsequently transferred to Immobilon membranes, and phosphorylated proteins were detected by autoradiography. Phosphoaminoacid analysis of phosphorylated proteins was performed as previously described (1, 21).

Labeling of cells with [^{32}P]orthophosphoric acid

Cells were washed with phosphate-free RPMI 1640 and cultured for 30 min at 37°C in phosphate-free medium. The cells were subsequently incubated in phosphate-free medium with carrier-free [^{32}P]orthophosphoric acid (DuPont-NEN, Boston, MA) at a concentration of 0.4 mCi/ml for 3 h. The ^{32}P-labeled cells were subsequently washed with phosphate-free RPMI medium, stimulated with 10^4 U/ml of IFN-α for the indicated times, and lysed in phosphorylation lysis buffer. The lysates were immunoprecipitated with the indicated Abs, washed five times in phosphorylation lysis buffer, and analyzed by SDS-PAGE.

Northern blot analysis

Cells were treated for 6 h with IFN-α, in the absence or presence of wortmannin (100 nM), which was added to the cultures 60 min before IFN-α treatment. Northern blots were subsequently performed with 30 μg total RNA extracted from treated and untreated cells.

Preparation of nuclear cell extracts

Nuclear extracts for mobility shift assays were prepared as follows. Cells were washed twice with ice cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF and once with hypotonic buffer. Following incubation for 10 min in hypotonic buffer at 10^8 cells/ml, cells were disrupted by repeated passage through a 25-gauge needle and centrifuged at 12,000 × g for 20 s. The supernatant (cytoplasmic fraction) was discarded. The pellet (crude nuclei) was suspended in hypotonic buffer and centrifuged at 7,000 × g for 5 min. The pellet (nuclear fraction) was incubated in high salt buffer at 2.5 × 10^8 cells/ml for 30 min, clarified by centrifugation at 12,000 × g for 20 min, and the supernatant was supplemented with 0.5% Triton X-100. Nuclear fractions yielded approximately 15 μg of protein/10^6 cells, based on the Bradford method for protein determination. (Bio-Rad Labs, Hercules, CA). Nuclear fractions were aliquoted and stored at −70°C. The hypotonic buffer contains: 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.9), 0.6 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM NaF, 0.6 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A. This buffer, containing 300 mM KCl and 20% glycerol constitutes high salt buffer.

Oligonucleotides

A double-stranded oligodeoxynucleotide, representing nucleotides −107 to −87 of the human 2′–5′ oligoadenylate synthetase gene, which contains a functional ISRE, was synthesized. The sequence was: CCTTCTGAGGC CACTAGAGCA. The oligonucleotide was synthesized with *SalI* compatible linkers at the 5′ terminus (TCGAC). Gel-purified oligonucleotides were mixed with their respective components, heated to 65°C for 15 min, and annealed at room temperature for 18 h.

Electrophoretic mobility shift assays (EMSA)

Ten micrograms of nuclear extracts from untreated or IFN-α-treated cells were analyzed using an EMSA. Briefly, extracts were incubated with or without double-stranded oligodeoxynucleotides corresponding to the 2′–5′ oligoadenylate synthetase ISRE, in the presence of 1 μg poly(dI:dC) · poly(dI:dC), in EMSA buffer for 30 min at room temperature (final volume 21 μl). Protein-DNA complexes were resolved by 4.5% PAGE using 0.5 × TBE (Tris, boric acid, EDTA) as running buffer. EMSA buffer contains 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA (pH 8.0), 4 mM Tris (pH 7.9), 0.5 mM DTT, and 10% glycerol.

Results

We initially performed experiments in which cells were incubated in the presence or absence of IFN-α, cell extracts were immunoprecipitated with an anti-IRS-1 Ab, and in vitro kinase assays were conducted on the anti-IRS-1 immunoprecipitates. A minimal amount of de novo phosphorylation of IRS-1 was detected in immunoprecipitates from control cells (Fig. 1, *A–C*). However, IFN-α treatment of cells stimulated strong phosphorylation of IRS-1 in vitro, suggesting that during IFN-α stimulation IRS-1 associates with an IFN-α-activated kinase(s). Phosphoaminoacid analysis of IRS-1 before and after IFN-α stimulation demonstrated that the protein is phosphorylated on tyrosine in an IFN-α-dependent manner (Fig. 1*D*). In addition to tyrosine phosphorylation, however, treatment of cells with IFN-α induced strong phosphorylation of IRS-1 on serine (Fig. 1*D*), suggesting that a serine kinase associates with and phosphorylates IRS-1.

To determine whether IRS-1 is phosphorylated on serine during treatment of intact cells with IFN-α, we performed studies using ^{32}P-labeled Daudi cells. Some baseline phosphorylation of IRS-1 was also detected under these conditions and increased during IFN-α stimulation (Fig. 2*A*). Phosphoaminoacid analysis of the protein demonstrated that the baseline phosphorylation of IRS-1 is on serine residues. After IFN-α stimulation, in addition to induction of tyrosine phosphorylation, the phosphoserine content of the protein also increased (Fig. 2*B*), demonstrating that IFN-α induces serine phosphorylation of IRS-1 in vivo.

Because IRS-1 has been previously shown to interact with the p85 subunit of the PI 3′-kinase during IFN-α stimulation (13), we sought to determine whether the serine phosphorylation of IRS-1 results from activation of the p110 catalytic subunit of this kinase. Wortmannin is a fungal metabolite that has been previously shown to selectively inhibit both the lipid (22) and serine (17, 18) kinase activities of the PI 3′-kinase. U-266 cells were treated with IFN-α in the presence or absence of wortmannin, and in vitro kinase assays were conducted on the αIRS-1 immunoprecipitates. The IFN-α-induced phosphorylation of IRS-1, which is mainly composed of phosphoserine, was partially inhibited in the presence of wortmannin (Fig. 3*A*), suggesting that its phosphorylation is PI 3′-kinase-dependent. The insulin-induced serine phosphorylation of IRS-1 was also inhibited by wortmannin (Fig. 3*A*), in agreement with previous reports (17, 18). On the other hand, wortmannin had no inhibitory effect on the IFN-α-induced tyrosine phosphorylation

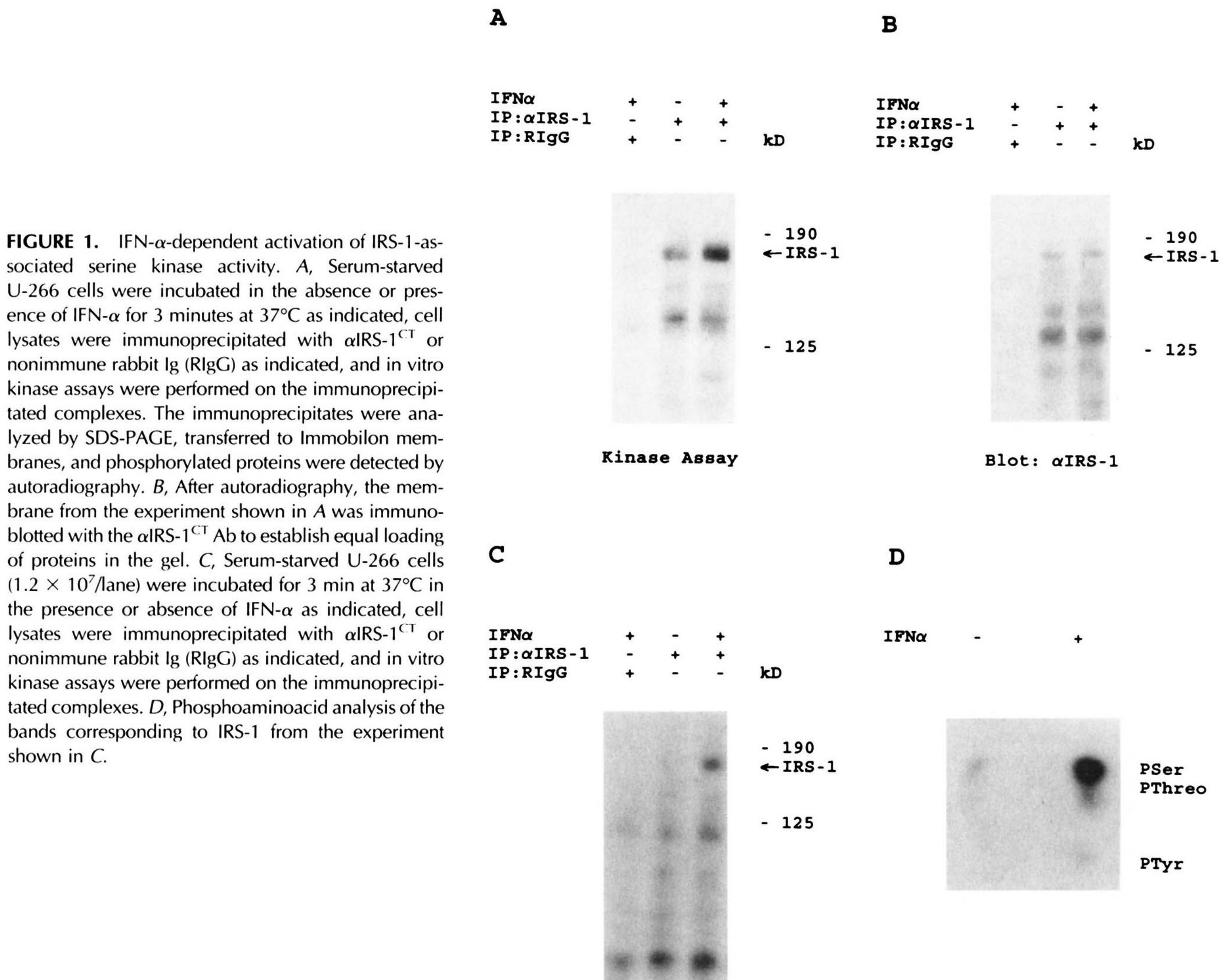


FIGURE 1. IFN-α-dependent activation of IRS-1-associated serine kinase activity. *A*, Serum-starved U-266 cells were incubated in the absence or presence of IFN-α for 3 minutes at 37°C as indicated, cell lysates were immunoprecipitated with αIRS-1^{CT} or nonimmune rabbit Ig (RIgG) as indicated, and in vitro kinase assays were performed on the immunoprecipitated complexes. The immunoprecipitates were analyzed by SDS-PAGE, transferred to Immobilon membranes, and phosphorylated proteins were detected by autoradiography. *B*, After autoradiography, the membrane from the experiment shown in *A* was immunoblotted with the αIRS-1^{CT} Ab to establish equal loading of proteins in the gel. *C*, Serum-starved U-266 cells (1.2×10^7/lane) were incubated for 3 min at 37°C in the presence or absence of IFN-α as indicated, cell lysates were immunoprecipitated with αIRS-1^{CT} or nonimmune rabbit Ig (RIgG) as indicated, and in vitro kinase assays were performed on the immunoprecipitated complexes. *D*, Phosphoaminoacid analysis of the bands corresponding to IRS-1 from the experiment shown in *C*.

of IRS-1 in intact cells (Fig. 3*B*), suggesting that it selectively inhibits the serine kinase that phosphorylates IRS-1.

We subsequently performed in vitro kinase assays on anti-p85 immunoprecipitates from U-266 cells. An 85-kDa phosphorylated protein, corresponding to phosphorylated p85, was seen in anti-p85 immunoprecipitates from unstimulated cells after incubation with [γ-^{32}P]ATP (Fig. 4*A*). After IFN-α stimulation, a 170-kDa phosphorylated protein corresponding to IRS-1 was also detectable in αp85 immunoprecipitates incubated with [γ-^{32}P]ATP (Fig. 4*A*). Pretreatment of cells with wortmannin resulted in partial inhibition of the phosphorylation of p85 and associated IRS-1 (Fig. 4, *A* and *B*), further suggesting that the IFN-α-induced serine phosphorylation of IRS-1 is regulated by the PI 3′-kinase.

Wortmannin also inhibited the phosphorylation of p85 and associated IRS-1 when added directly on p85 immunoprecipitates from IFN-α-stimulated cells (Fig. 5, *A* and *B*), strongly suggesting that its inhibitory effect is direct.

It is well established that in the doses used here wortmannin exhibits selectivity for the PI 3′-kinase (22, 23) and does not affect activation of the insulin receptor tyrosine kinase (17). Nevertheless, and to exclude the possibility that its inhibitory effect on IFN-α serine phosphorylation of IRS-1 results from inhibition of upstream tyrosine kinases, we performed studies to determine whether wortmannin inhibits activation of the IFN-α-dependent Tyk-2 and Jak-1 kinases. After preincubation in the presence or absence of wortmannin, cells were stimulated with IFN-α, and the phosphorylation/activation of Tyk-2 and Jak-1 were determined. Figure 6, *A* and *B*, demonstrates that pretreatment of cells with wortmannin did not abrogate IFN-α-dependent activation of Tyk-2 or Jak-1, excluding such a possibility. Furthermore, in studies to determine whether the p85 subunit of the PI 3′-kinase associates with Jak kinases or the type I IFNR, we were unable to demonstrate any interaction between p85 and Tyk-2 and Jak-1 or the α and β_S subunits of the type I IFN receptor (Fig. 6*C*), suggesting that the PI 3′-kinase does not interact with IFN-α-signaling components upstream of IRS-1.

A recent report has demonstrated that the Map kinase is activated by IFN-β (24). It is has been also suggested that the PI 3′-kinase exhibits a regulatory role on the activation of the Map kinase (25–27) and the Ras pathway (28). Our findings, taken together with the results of these studies, raised the possibility that one of the biochemical functions of the PI 3′-kinase in type I IFN signaling may be regulation of the Map kinase and downstream pathways. We therefore sought to determine whether wortmannin inhibits the type I IFN activation of the Map kinase. U-266 cells were treated with IFN-β in the absence or presence of wortmannin, and total cell lysates were immunoblotted with an anti-Map kinase Ab that recognizes the active/phosphorylated forms of Erk-1 and Erk-2. IFN-β treatment of cells resulted in activation of the Map kinase, and such activation was partially inhibited in the presence of wortmannin (Fig. 7*A*). Stripping and reblotting the

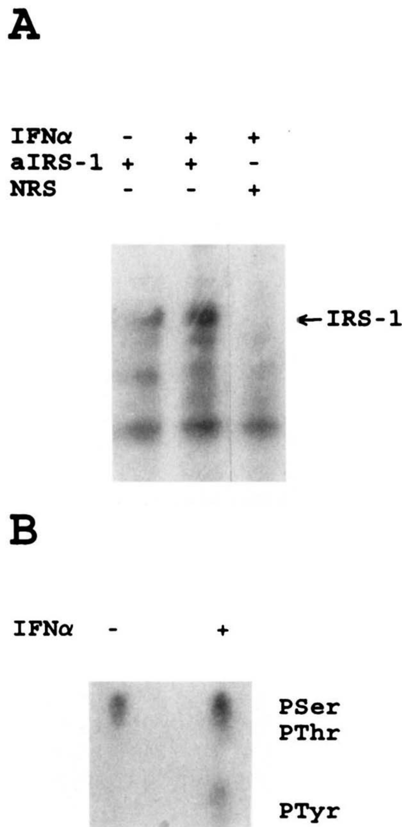


FIGURE 2. IFN-α induces serine phosphorylation of IRS-1 in intact cells. *A*, ^{32}P-labeled Daudi cells (1×10^8/lane) were incubated in the presence or absence of IFN-α for 15 min at 37°C as indicated. The cells were lysed, and the cell lysates were immunoprecipitated with either an αIRS-1 Ab or nonimmune rabbit serum as indicated, and analyzed by SDS-PAGE. *B*, Phosphoaminoacid analysis of IRS-1 before and after IFN-α stimulation from the experiment shown in *A*.

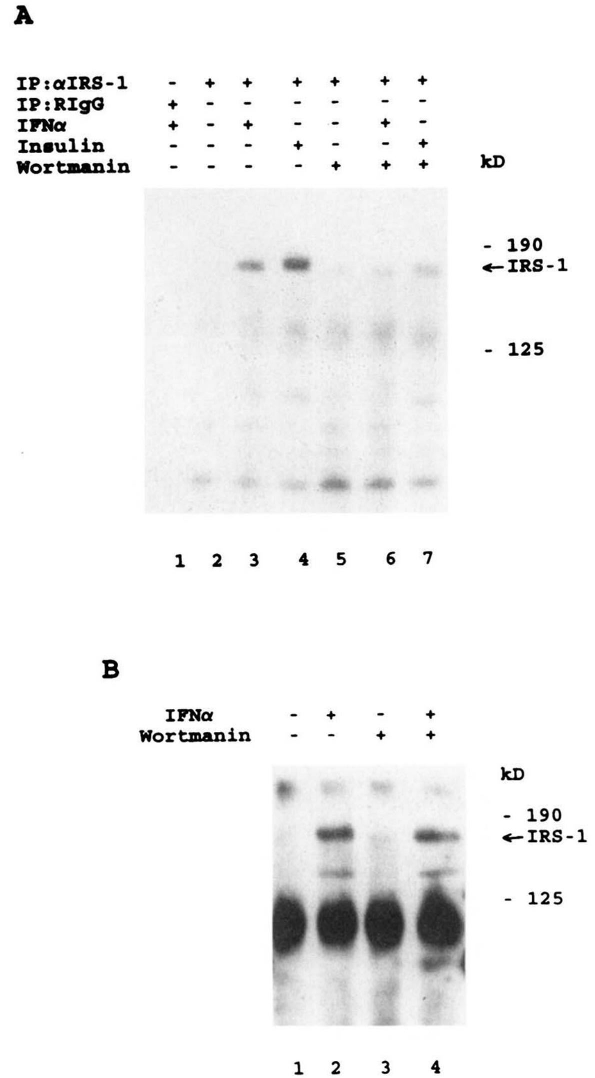


FIGURE 3. Inhibition of IFN-α-dependent serine phosphorylation of IRS-1 by the PI 3′-kinase inhibitor wortmannin. *A*, Serum-starved U-266 cells (2.2×10^7/lane) were preincubated for 20 min at 37°C in the presence or absence of wortmannin (100 nM) as indicated. The cells were subsequently treated, in the continuous presence or absence of wortmannin, with IFN-α for 3 min as indicated. Cell lysates were immunoprecipitated with either αIRS-1^{CT} (*lanes 2–7*) or nonimmune RIgG (*lane 1*), and in vitro kinase assays were carried out on the immunoprecipitates. *B*, Serum-starved Daudi cells (1.6×10^7/lane) were preincubated for 30 min in the presence or absence of wortmannin (150 nM) as indicated. The cells were subsequently treated, in the continuous presence or absence of wortmannin, with IFN-α for 5 min as indicated. Cell lysates were immunoprecipitated with αIRS-1^{CT} (*lanes 1–4*), and proteins were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine.

same blot with an anti-Erk-2 Ab (that also cross-reacts with Erk-1) demonstrated that the amounts of Map kinase present in the lysates from cells treated in the presence or absence of wortmannin were similar (Fig. 7*B*). Similar results were obtained using IFN-α (data not shown). Thus, wortmannin inhibits the IFN-induced activation of the Map kinase, suggesting that the PI 3′-kinase may act as an upstream regulator of Map kinase in type I IFN signaling. Because the activation of the Map kinase occurs via phosphorylation on threonine and tyrosine residues, it is likely that intermediate signaling molecules between the PI 3′-kinase and the Map kinase are involved.

The previous study that demonstrated activation of the Map kinase by IFN-β had also suggested that the function of the Map kinase may regulate the Stat pathway and IFN-induced gene expression (24). We therefore sought to determine whether treatment of cells with wortmannin also results in inhibition of Stat activation and gene transcription. We studied the effect of wortmannin on the IFN-α-induced activation of ISGF3, which is composed of the ISGF3α complex (Stat-2, Stat-1α, and Stat-1β) and ISGF3γ(p48), and binds to IFN-stimulated response elements (ISREs) to initiate gene transcription. In both U-266 and Daudi cells studied, treatment of cells with wortmannin did not inhibit formation of the ISGF3 DNA-binding complex (Fig. 8*A*). Indeed, we observed no effect also on SIE binding Stat activity (data not shown). These results suggest that phosphorylation/activation of Stat-1, Stat-2, and Stat-3 are not affected by wortmannin treatment. To directly determine the effect of wortmannin on IFN-induced gene expression, we studied its effect on the expression of the ISG-15 gene, which is dependent on the Jak-Stat pathway. wortmannin failed to inhibit IFN-induced RNA expression for the ISG-15 gene (Fig. 8, *B* and *C*), confirming that activation of the PI 3′-kinase does not play a regulatory role on the Stat pathway.

Discussion

Considerable advances have been made in our understanding of the signaling events elicited during binding of IFN-α to the multisubunit type I IFN receptor. It is now well established that the Tyk-2 and Jak-1 tyrosine kinases are associated with components of the type I

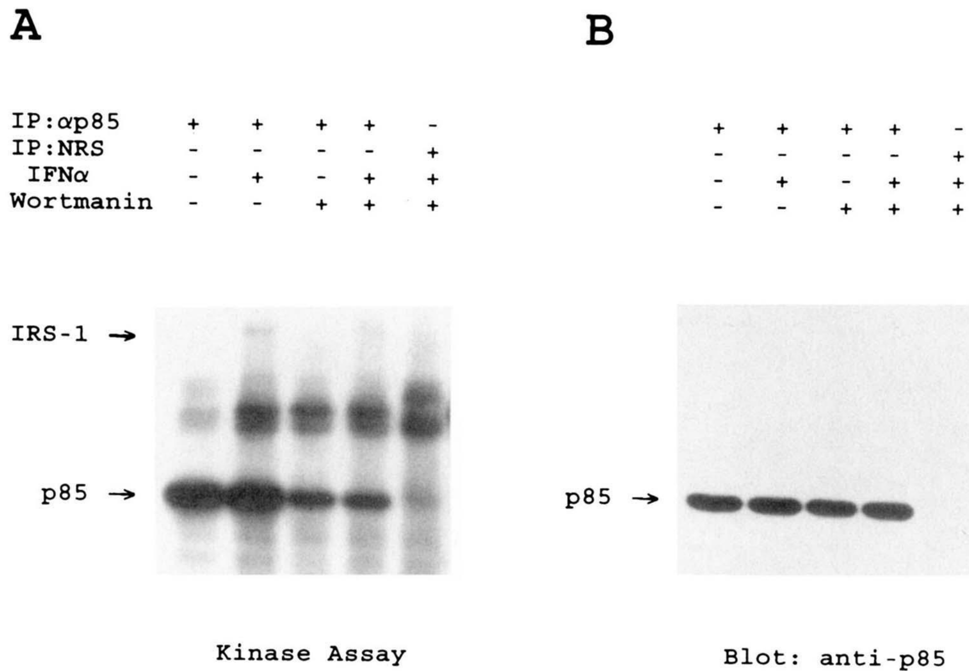


FIGURE 4. Inhibition of the serine kinase activity of the PI 3′-kinase by wortmannin. *A*, Serum-starved U-266 cells (3.5×10^7/lane) were preincubated for 20 min at 37°C in the presence or absence of wortmannin (100 nM) as indicated. The cells were subsequently treated, in the continuous presence or absence of wortmannin, with IFN-α for 5 min as indicated. Cell lysates were immunoprecipitated with either a polyclonal Ab against p85 or normal rabbit serum as indicated, and in vitro kinase assays were carried out on the immunoprecipitates. Proteins were analyzed by SDS-PAGE, transferred to Immobilon, and visualized by autoradiography. *B*, After autoradiography exposure, the membrane from the experiment shown in *A* was immunoblotted with a mAb against p85α, to establish that equal amounts of p85α were present in the αp85 immunoprecipitates.

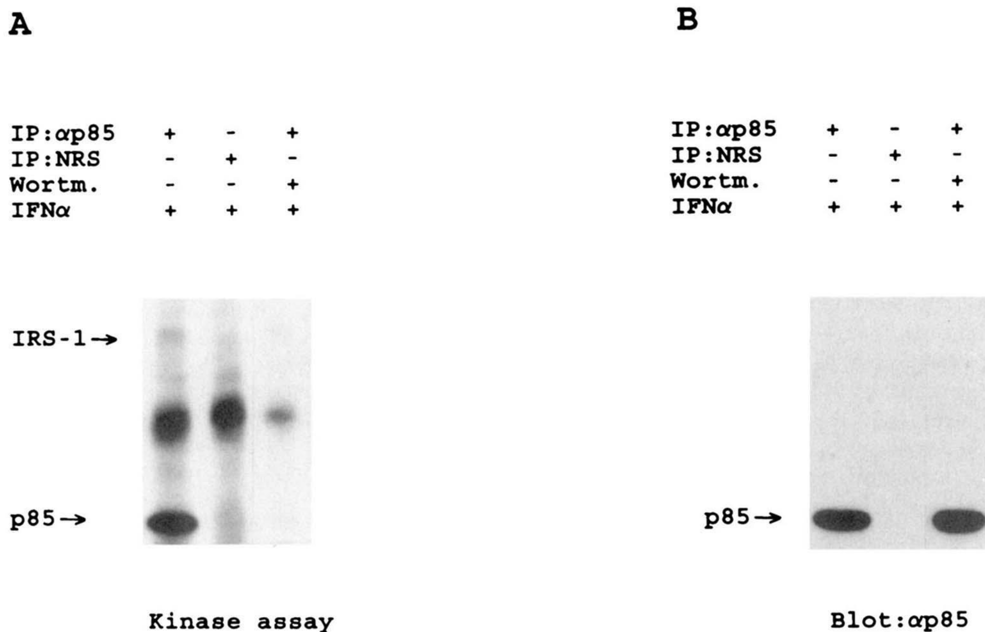


FIGURE 5. Inhibition of the IFN-α-activated PI 3′-kinase serine kinase by wortmannin in vitro. *A*, Serum-starved U-266 cells (3.6×10^7/lane) were treated with IFN-α for 5 min at 37°C as indicated. Cell lysates were immunoprecipitated with the indicated Abs, and 100 nM of wortmannin was added directly to the beads for 20 min at room temperature as indicated, before carrying out an in vitro kinase assay. Proteins were analyzed by SDS-PAGE, transferred to Immobilon, and visualized by autoradiography. *B*, After autoradiography exposure, the membrane from the experiment shown in *A* was immunoblotted with a mAb against p85α to establish that equal amounts of p85α were present in the αp85 immunoprecipitates.

IFN receptor (19, 29–31). During engagement of the type I IFN receptor, these kinases are activated and regulate activation of the Stat- (8–10, 32) and IRS-signaling pathways (13, 14). Thus, tyrosine kinases appear to play a critical role in IFN-α signaling (32–34). There are several lines of evidence, however, indicating that serine phosphorylation also plays an important role in type I IFN signaling. Previous studies have shown that various IFN-α-signaling elements, including the α subunit of the type I IFN receptor (1), the Vav protooncogene (12), and Stat-1 (24), exhibit constitutive and/or IFN-α-induced serine phosphorylation, suggesting that they are substrates for

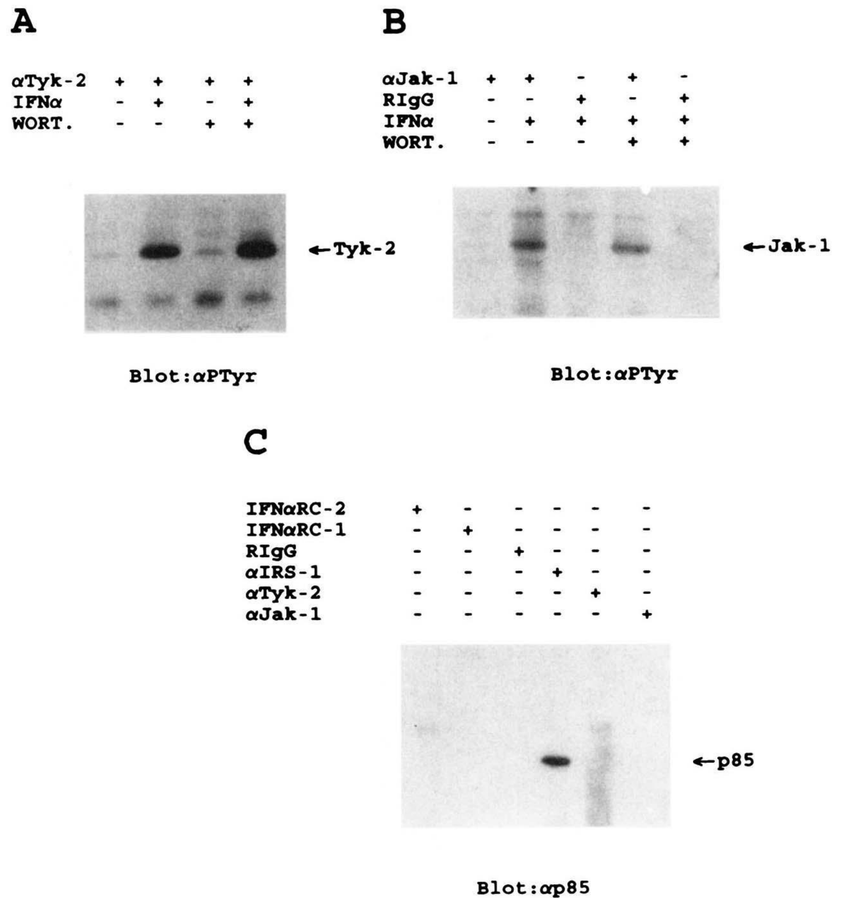


FIGURE 6. Wortmannin does not inhibit the IFN-α-dependent phosphorylation of Tyk-2 and Jak-1. *A*, HSB-2 cells (3.5 × 10^7/lane) were preincubated for 20 min at 37°C in the presence or absence of wortmannin (100 nM) as indicated. The cells were subsequently treated, in the continuous presence or absence of wortmannin, with IFN-α for 5 min as indicated. The cells were lysed, and cell lysates were immunoprecipitated with an Ab against Tyk-2. Proteins were anayzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine. *B*, HSB-2 cells (4.3 × 10^7/lane) were preincubated for 20 min at 37°C in the presence or absence of wortmannin (100 nM) as indicated. The cells were subsequently treated, in the continuous presence or absence of wortmannin, with IFN-α for 5 min as indicated. The cells were lysed, and cell lysates were immunoprecipitated with either an Ab against Jak-1 or normal rabbit IgG (RIgG) as indicated. Proteins were analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. *C*, Serum-starved U-266 cells (2.4 × 10^7/lane) were treated with IFN-α for 5 min at 37°C as indicated. Cell lysates were immunoprecipitated with the IFN-αRC-2 (β_S subunit of the type I IFNR) or IFN-αRC-1 (α subunit of the type I IFNR) Abs or normal RIgG or αIRS-1^{CT} or αTyk-2 or αJak-1 as indicated. Proteins were analyzed by SDS-PAGE and immunoblotted with a mAb against p85α.

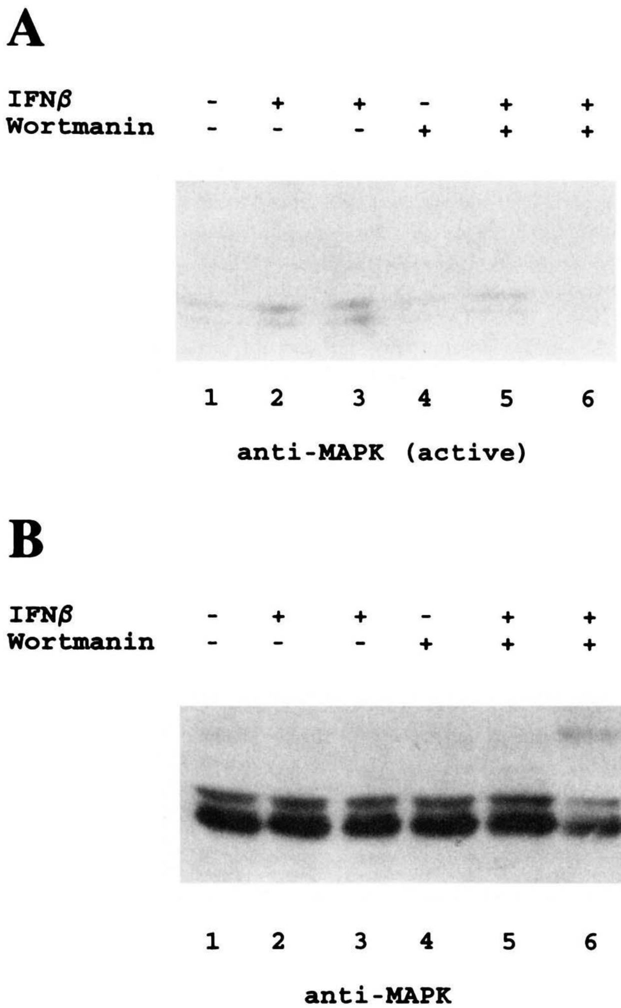
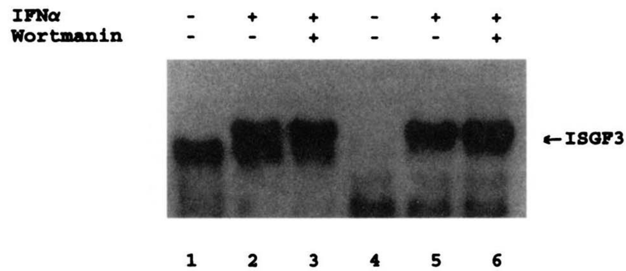
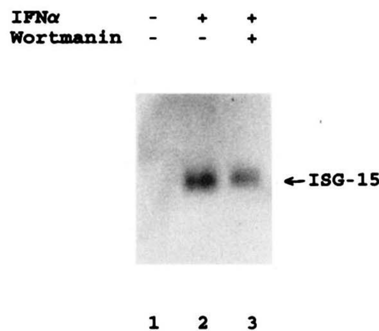


FIGURE 7. Inhibition of activation of Map kinase by wortmannin. *A*, U-266 cells were preincubated for 60 min at 37°C in the presence or absence of wortmannin (100 nM) and were subsequently either not treated with IFN-β (*lanes 1* and *4*) or treated with IFN-β for 10 min (*lanes 2* and *5*) or 20 min (*lanes 3* and *6*). Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an Ab that recognizes the phosphorylated forms of Map kinase (New England Biolabs). *B*, The blot shown in *A* was stripped and reprobed with an anti-Map kinase Ab (anti-Erk-2, Transduction Laboratories).





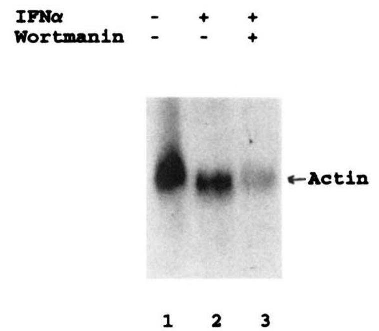


FIGURE 8. Effect of wortmannin on Stat activation and ISG transcription. *A*, U-266 (*lanes 1–3*) or Daudi (*lanes 4–6*) cells were pre-incubated in the presence or absence of wortmannin (100 nM) for 30 min at 37°C as indicated, and were subsequently treated with IFN-α for 10 min as indicated. Nuclear extracts were reacted with 40,000 cpm of a ^{32}P-labeled ISRE, and complexes were resolved by native gel electrophoresis and visualized by autoradiography. *B*, Daudi cells were incubated for 60 min at 37°C in the presence or absence of 100 nM of wortmannin as indicated. The cells were subsequently incubated at 37°C for 6 h in the presence or absence of IFN-α (1000 U/ml) as indicated, in the continuous presence of wortmannin for the cells that were pretreated with the PI 3′-kinase inhibitor. After isolation of total RNA, a northern blot was hybridized with an ISG-15 gene probe. *C*, The same blot as shown in *B* was stripped and hybridized with a chicken α-actin probe as an internal control.

serine kinase activity. Furthermore, maximal activation of Stat-3 and Stat-1 in response to other cytokines requires both serine and tyrosine phosphorylation (35).

The PI 3′-kinase lipid kinase participates in various signaling cascades and regulates many important biologic responses (36). The p110 catalytic subunit of the PI 3′-kinase also possesses serine kinase activity (15, 16), which has been previously shown to constitutively phosphorylate its p85 regulatory subunit (17, 18). During insulin stimulation, the PI 3′-kinase serine kinase is activated and phosphorylates IRS-1 (17, 18). It has been proposed that such phosphorylation may change the conformation of IRS-1 and alter its affinity for binding signaling molecules, or generate signals that along with PI-3,4-P_2, or PI-3,4,5-P_3 are necessary to regulate downstream pathways (17). The recent finding, that wortmannin inhibits the serine kinase activity of PI 3′-kinase (17), raises the intriguing possibility that several biologic activities of insulin that have been previously ascribed to the function of the lipid PI 3′-kinase may also require a functional serine kinase or the serine kinase activity alone.

In the current study, we demonstrate that the serine kinase activity of the PI 3′-kinase is activated by IFN-α, and we identify one of its putative substrates as the IRS-1. Although our data strongly suggest that the serine kinase activity of the PI 3′-kinase is responsible for the serine phosphorylation of IRS-1, we cannot absolutely exclude the possibility that another serine kinase downstream of the PI 3′-kinase regulates IRS-1 phosphorylation.

Recent studies have shown that type I IFN stimulation results in activation of the Map kinase, and this event exhibits a regulatory role on the activation of the Stat pathway (24). It is also known that in other systems the PI 3′-kinase exhibits a regulatory role on the activation of the Map kinase (25–27). In the current study we confirmed that IFN-β activates the Map kinase and also demonstrated that such activation is partially inhibited by wortmannin, suggesting that the serine and/or lipid kinase activities of the PI 3′-kinase participate in such regulation. However, wortmannin failed to inhibit the formation of the ISGF-3 complex and RNA expression for the ISG-15 gene, events that depend on Stat protein phosphorylation. Thus, it is unlikely that the PI 3′-kinase serine kinase regulates serine phosphorylation of Stat proteins, and appears to exhibit selectivity for IRS-1. These results are also consistent with our recent data, using IRS-defective cells, that established that the Stat pathway is distinct from the IRS pathway.[4]

Type I IFNs are pleiotropic cytokines that exhibit multiple biologic effects, including antiviral and antiproliferative activities. It is now well established that the transcriptional activity of Stat proteins regulates type I IFN-induced gene expression and antiviral activities (37). However, the mechanisms that mediate the antiproliferative effect of type I IFNs are not as well elucidated. Activation of the Map kinase is known to engage downstream pathways regulating cell growth and differentiation in other systems, and our data provide evidence that in the type I IFN system, its activity may be regulated by the PI 3′-kinase. Further studies to determine the precise role that the PI 3′-kinase and Map-kinase serine kinases may play in IFN-induced cell growth inhibition and differentiation are warranted and may provide

[4] S. Uddin, E. N. Fish, D. Sher, C. Gardziola, O. R. Colamonici, M. Kellum, P. M. Pitha, M. F. White, and L. C. Platanias. The insulin receptor substrate (IRS)-signaling system mediates common and distinct signals during IFN-α or insulin stimulation. *Submitted for publication.*

valuable information on the mechanisms by which these cytokines mediate their antitumor effects.

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