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Interferon-Dependent Activation of the Serine Kinase PI 3′-Kinase Requires Engagement of the IRS Pathway but Not the Stat Pathway

Shahab Uddin,*,† Beata Majchrzak,‡ Pu-Chen Wang,*,† Sanjiv Modi,*,† Mohammad K. Khan,*,† Eleanor N. Fish,‡ and Leonidas C. Platanias*,†,[1]

**Section of Hematology–Oncology, Department of Medicine, University of Illinois at Chicago, Chicago, Illinois; †West Side VA Hospital, Chicago, Illinois 60607; and ‡Division of Cell and Molecular Biology, Toronto General Research Institute University Health Network, and Department of Immunology, University of Toronto, Toronto, Ontario M5G 2M1, Canada*

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Several signaling pathways are activated by interferon α (IFNα) in hematopoietic cells, including the Jak-Stat and the insulin receptor substrate (IRS) pathways. It has been previously shown that IFNα activates the phosphatidylinositol (PI) 3′-kinase via an interaction of the p85 subunit of PI 3′-kinase with IRS proteins. Other studies have proposed that Stat-3 also functions as an adapter for p85. We sought to identify the major pathway that regulates IFNα activation of the PI3′-kinase in hematopoietic cells. Our data demonstrate that IFNα induces the interaction of p85 with IRS-1 or IRS-2, but not Stat-3, in various hematopoietic cell lines in which IRS-1 and/or IRS-2 and Stat-3 are activated by IFNα. In addition, inhibition of PI 3′-kinase activity by preincubation of cells with the PI 3′-kinase inhibitor LY294002 does not affect IFN-dependent formation of SIF complexes that contain Stat-3. To determine whether phosphorylation of tyrosine residues in the IFN receptor is required for activation of the PI 3′-kinase, we performed studies using mouse L929 fibroblasts transfected with mutated human IFNAR1 and/or IFNAR2 subunits of the Type I IFN receptor, lacking tyrosine phosphorylation sites. The serine kinase activity of the PI-3K was activated by human IFNα in these cells, suggesting that phosphorylation of the Type I IFN receptor is not essential for PI3K activation. We then determined whether IFNα activates the Akt kinase, a known downstream target for PI 3′-kinase that mediates antiapoptotic signals. Akt was activated by insulin or IGF-1, but not IFNα, in the IFNα-sensitive U-266 myeloma cell line. Altogether, our data establish that the IRS pathway and not the Stat pathway, is the major pathway regulating engagement of PI 3′-kinase in hematopoietic cells. Furthermore, the selective activation of Akt by insulin/IGF-1 suggests the existence of distinct regulatory activities of PI3′-kinase in growth factor versus interferon signaling.

It is well established that two tyrosine kinases of the Janus family, Tyk-2 and Jak-1, are constitutively associated with components of the Type I interferon receptor and are rapidly phosphorylated and activated in response to binding of Type I interferons to the receptor (reviewed in 1–3). The IFNα-dependent activation of these tyrosine kinases results in the engagement of several signaling elements and cascades, including the Jak-Stat pathway (reviewed in 1–3), the IRS pathway (4–7) and the CrkL pathway (8, 9) to mediate the diverse biological effects of interferons on target cells.

Although the role of tyrosine kinases of the Janus family is well established and characterized, the role of serine/threonine kinases in the interferon signaling system is not well defined. The phosphatidylinositol 3′-kinase plays a critical role in signaling for various ligands (reviewed in 10, 11). Two distinct subunits form the functional PI 3′-kinase, the p85 regulatory subunit that contains SH2 and SH3 domains and mediates its interactions with other signaling elements, and the p110 catalytic subunit that contains the functional kinase domain (10, 11). Previous studies have established that the phosphatidylinositol (4) and serine kinase activities (12) of the PI 3′-kinase are activated during the association of the p85 catalytic subunit with tyrosine phosphorylated IRS-1 in response to Type I IFN treatment. In addition, the p85 subunit of the PI 3′-kinase has been shown to interact with IRS-2 in an IFNα-dependent manner (5), indicating that this member of the IRS family of proteins also regulates engagement of this pathway in IFN signal-

[1] To whom correspondence should be addressed at Section of Hematology–Oncology, University of Illinois at Chicago, MBRB, MC-734, Rm. 3150, 900 S. Ashland Ave., Chicago, IL 60607-7173. Fax: (312) 413-7963. E-mail: Lplatani@UIC.Edu.

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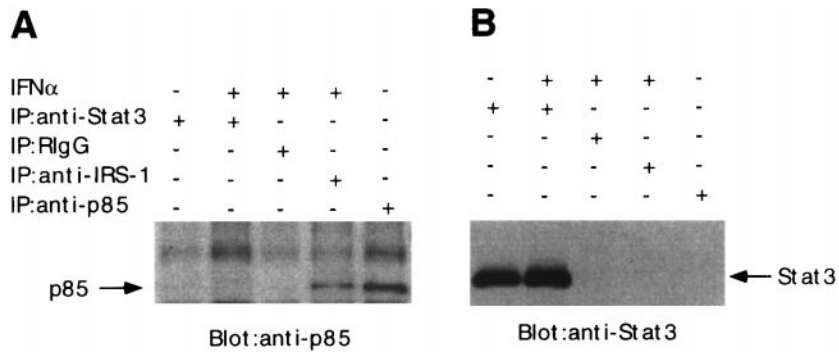

FIG. 1. Association of the p85 subunit of the PI 3′ kinase with IRS-1, but not Stat-3, in Daudi cells. (A) Daudi cells were incubated in the presence or absence of IFNα for 5 min as indicated. Cell lysates were immunoprecipitated with the indicated antibodies and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody against p85α. (B) The blot shown in A was stripped and reprobed with an antibody against Stat-3.

ing. Other studies have also suggested that Stat-3 may play the role of an adapter, linking the PI 3′-kinase to the IFNaR1 chain of the Type I IFN receptor (13), raising the possibility of an interplay between the Stat and PI3K pathways in interferon signaling.

In the present study we performed experiments to identify the major pathway that regulates IFNα-dependent activation of the PI 3′-kinase in cells of hematopoietic origin and to determine whether tyrosine phosphorylation of the Type I IFN receptor is required for its activation. In addition, we sought to determine whether the Akt kinase, a known downstream element of the PI 3′-kinase pathway (14), is activated by IFNα or insulin/IGF-1 in cells of hematopoietic origin. Our data establish that the IRS pathway and not the Stat pathway is the major signaling cascade regulating engagement of the PI 3′-kinase in response to IFNs and demonstrate that tyrosine phosphorylation of the Type I IFN receptor is not required for activation of the serine kinase activity of the PI 3′-kinase. Furthermore, they establish that, despite the activation of the PI 3′-kinase by IFNα, the Akt kinase is not activated, demonstrating that antiapoptotic signals downstream of the PI 3′-kinase are activated selectively by growth factors but not interferons.

MATERIALS AND METHODS

Cells and reagents. The human U-266 (multiple myeloma), Daudi (lymphoblastoid) and KG-1 (acute myeloid leukemia) cell lines were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum and antibiotics. The L929 transfectants with the different mutants of the Type I IFN receptor subunits were provided by Dr. Oscar Colamonici (Univ. of Illinois at Chicago, Chicago, IL) and were grown as previously described (16). Human recombinant IFNα was provided by Hoffmann Laroche. Human recombinant IFNα was provided by Biogen Inc. (Cambridge, MA). IFNα consensus was provided by Amgen Inc. The polyclonal antibodies against IRS-1, IRS-2 and p85 (4–6) were provided by Dr. M. F. White (Joslin Diabetes Center, Boston, MA). The antiphosphotyrosine monoclonal antibody (4G-10) and a monoclonal antibody against the p85 subunit of the PI 3′-kinase were obtained from Upstate Biotechnology. An antibody against PKB/Akt was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitations and immunoblotting. Cells were incubated at 37°C in the presence or absence of 10^4 U/ml of IFNα as indicated. Cell lysis, immunoprecipitation and immuno-blotting using the ECL method were performed essentially as previously described (4, 5).

In vitro kinase assays and phosphoamino acid analysis. These assays were performed essentially as previously described (12).

Gel shift analysis. Preparation of nuclear extracts and gel shift analysis were performed essentially as in previous studies (9, 12). The sequence of the oligonucleotide, corresponding to an SIE element, that was synthesized and used was 5′-ATTTCCCGTAAATCCC-3′.

PKB/Akt kinase assays. Cells were incubated for the indicated times in the presence or absence of IFNα. Cell lysates were immunoprecipitated with an antibody against PKB/AKT and *in vitro* kinase assays were performed using histone 2B as an exogenous substrate, as previously described (15).

RESULTS AND DISCUSSION

We determined whether Stat-3 associates with p85 in IFNα-sensitive cells, in which we have previously shown IFNα-dependent activation of IRS-1 (Daudi) (4), IRS-2 (KG-1) (5), or both IRS-1 and IRS-2 (U-266) (5). Figure 1 shows an experiment in which Daudi cells were incubated for 5 min in the presence or absence of IFNα, the cells were lysed, and cell lysates were immunoprecipitated with antibodies against IRS-1 or Stat-3, prior to SDS-PAGE analysis and immunoblotting with an anti-p85 antibody. Consistent with our previous findings (4), the p85 subunit of the PI 3′-kinase associated with IRS-1 immunoprecipitated from lysates of IFNα-treated cells (Fig. 1). However, we could not detect association of p85 with Stat-3 in these cells (Fig. 1). In a similar manner, when KG-1 cells were studied, p85 associated with IRS-2, which is tyrosine phosphorylated in an IFNα-dependent manner (5), but not Stat-3 (Figs. 2A and 2B). We also performed similar studies with U-266 cells, which express both

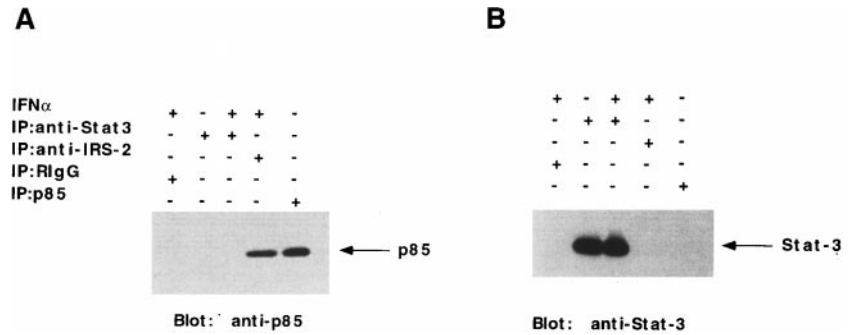

FIG. 2. Association of the p85 subunit of the PI 3′ kinase with IRS-2, but not Stat-3, in KG-1 cells. (A) KG-1 cells were incubated in the presence or absence of IFNα for 5 min as indicated. Cell lysates were immunoprecipitated with the indicated antibodies and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody against p85α. (B) The blot shown in A was stripped and reprobed with an antibody against Stat-3.

IRS-1 and IRS-2, and in which Stat-3 is also tyrosine phosphorylated and activated in response to Type I IFN treatment (Figs. 3A–3C). Consistent with our findings in the KG-1 and Daudi hematopoietic cell lines, we failed to detect an association of p85 with the IFNα-phosphorylated/activated form of Stat-3. Furthermore, in studies using the specific PI 3′-kinase inhibitor LY294002, we found that inhibition of the PI 3K activity does not block Stat-3 activation/DNA binding activity (Fig. 4). In a similar manner, another PI3′-kinase inhibitor, wortmannin, also did not block DNA binding of SIF complexes, consistent with our previous findings (12). Taken altogether, these data establish that Stat-3 does not play a critical role in regulating engagement of the p85 subunit of the PI 3′-kinase in IFNα signaling and that IRS-proteins provide the major pathway for the engagement of the PI3′-kinase.

We subsequently sought to determine whether phosphorylation of tyrosine residues in the Type I IFN receptor, including tyrosines Y^{527} and Y^{538} in IFNAR1, that have been suggested to engage Stat-3 (13), is required for activation of the PI 3′-kinase by IFNα. For these studies, we used mouse L929 cells transfected with either a human IFNAR1 subunit lacking all tyrosine phosphorylated sites, along with wild type

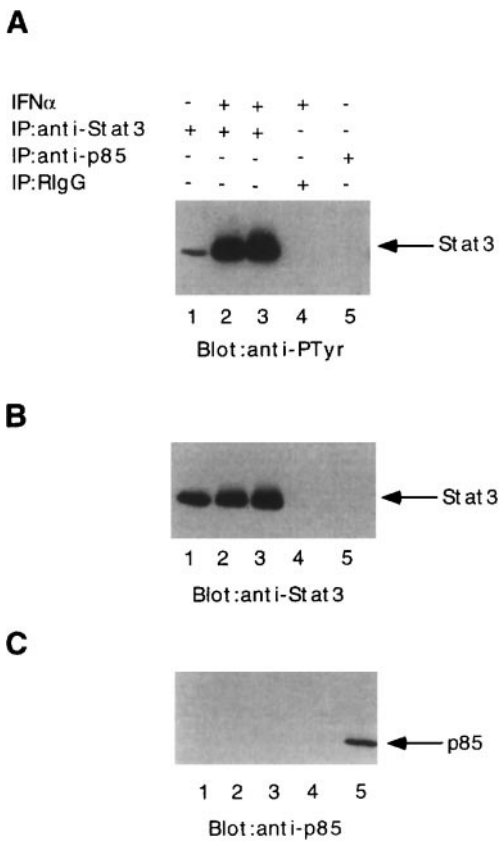

FIG. 3. Stat-3 does not interact with p85 in U-266 cells. (A) U-266 cells were either not treated with IFNα (lanes 1 and 5) or treated with IFNα for 5 min (lanes 2 and 4) or 15 min (lane 3). Cell lysates were immunoprecipitated with the indicated antibodies and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody against phosphotyrosine. (B) The blot shown in A was stripped and reprobed with an antibody against Stat-3. (C) The same blot was stripped again and reprobed with a monoclonal antibody against p85α.

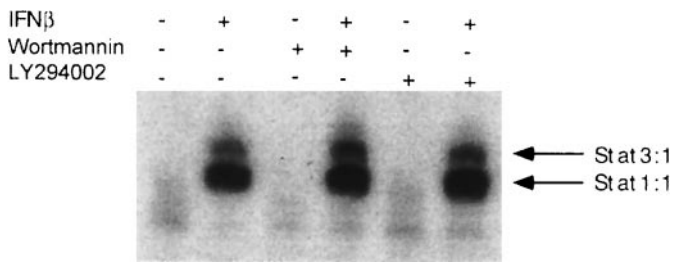

FIG. 4. Inhibition of PI 3′ kinase activity does not block activation of Stat-3 and formation of SIF complexes. Daudi cells were incubated in the presence or absence of 50 μM LY294002 or 100 nM wortmannin for 30 min at 37°C as indicated and then treated for 10 min in the presence or absence of IFNβ as indicated, in the continuous presence or absence of the PI 3′-kinase inhibitor. Nuclear extracts were reacted with a ^{32}P-labeled oligonucleotide specific for SIE. The resultant complexes were resolved using 4.5% native PAGE and visualized by autoradio-graphy.

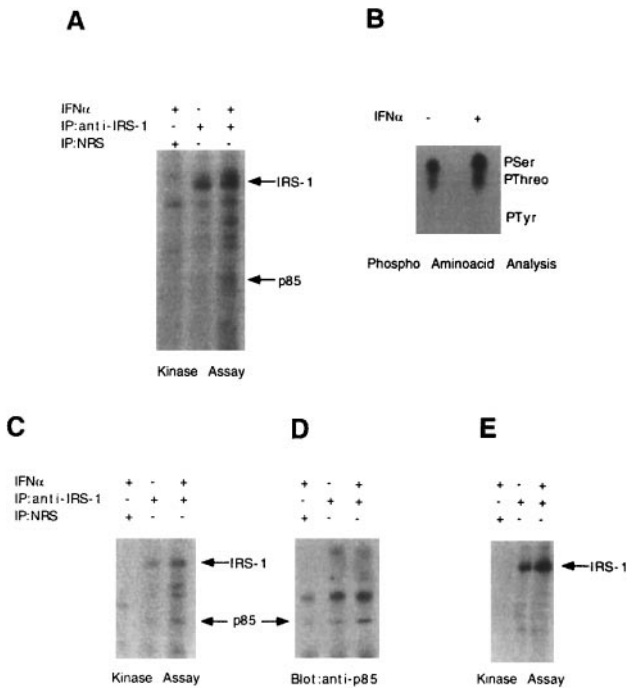

FIG. 5. The PI 3′-kinase serine kinase does not require tyrosine phosphorylation sites in the Type I interferon receptor for its activation. (A) Mouse L929 fibroblasts transfected with a mutated human IFNAR1 subunit and a human wild-type IFNAR2c (LαYF526βLwt) were incubated in the presence or absence of IFNα as indicated. Cell lysates were immunoprecipitated with the indicated antibodies and subjected to an *in vitro* kinase assay. After SDS-PAGE analysis the proteins were transferred to immobilon membranes and phosphorylated proteins were detected by autoradiography. (B) Phospho aminoacid analysis of the proteins corresponding to phosphorylated IRS-1 from the experiment shown in A. (C) Mouse L929 fibroblasts transfected with a mutated human IFNAR1 subunit and a human wild type IFNAR2c (LαYF526βLwt) were incubated in the presence or absence of IFNα as indicated. Cell lysates were immunoprecipitated with the indicated antibodies and subjected to an *in vitro* kinase assay. (D) The membrane from the experiment shown in C was immunoblotted with a monoclonal antibody against p85α. (E) Mouse L929 fibroblasts transfected with a mutated human IFNAR1 subunit and a mutated human IFNAR2c subunit (LαYFβL346YF) were incubated with human IFNα as indicated. Cell lysates were immunoprecipitated with the indicated antibodies and subjected to an *in vitro* kinase assay. After SDS-PAGE analysis the proteins were transferred to Immobilon membranes and phosphorylated proteins were detected by autoradiography.

IFNAR2c (LαYF526βLwt) or a mutated IFNAR1 subunit and a mutated IFNAR2c subunit also lacking all tyrosines (LαYFβL346YF) (16). As shown in Figs. 5A–5D, the serine kinase activity of the PI3′-kinase, which uses IRS-1 as an *in vivo* substrate (12), was activated in an IFNα-dependent manner, indicating that tyrosine phosphorylation of the IFNAR1 subunit is not required for activation of the PI 3′-kinase. In a similar manner, the serine kinase activity of p85 was activated in cells expressing both IFNAR1 and IFNAR2 without tyrosines (LαYFβL346YF) (16) (Fig. 5E). Taken altogether, the studies with the different receptor mutants establish that the PI 3′-kinase does not require tyrosine phosphorylation sites in the receptor complex for its activation.

It is well established that, in addition to interferons, insulin, insulin-like growth factor I and several cytokines and growth factors activate the PI3′-kinase pathway (reviewed in 11, 17). It is of interest, however, that in contrast to interferons, all these growth factors induce growth promoting and antiapoptotic signals. A major pathway downstream of the PI 3′-kinase in growth factor signaling involves activation of the PKB/Akt kinase that mediates antiapoptotic effects (14, 18–22). We sought to determine whether, in hematopoietic cells, PKB/Akt is activated in response to insulin/IGF-1 or interferon treatment. As shown in Fig. 6, insulin treatment of U-266 myeloma cells, which express functional insulin receptors (23), resulted in activation of the Akt kinase. Similarly, treatment with IGF-1 also resulted in activation of the Akt kinase in these cells (data not shown). On the other hand, IFNα treatment did not induce Akt activation (Fig. 6), demonstrating that the Akt kinase is not a downstream effector of the PI 3′-kinase in IFNα signaling.

We have previously shown that the phosphatidylinositol- and serine-kinase activities of the PI 3′-kinase are rapidly activated in an IFNα-depedent manner during the interaction of the p85 subunit of the PI 3′-kinase with IRS-1 (5, 12) and that IRS-1 itself is a substrate for the serine catalytic activity of the PI 3′-kinase (12). More recently, it was reported by others that Stat-3 may also participate in a pathway regulating activation of the PI 3′-kinase (13). In the present study we sought to identify whether the Stat-3 or the IRS-pathway is the major pathway regulating engagement of the PI 3′-kinase in Type I interferon signaling and whether tyrosine phosphorylation of the Type I interferon receptor is required for such an activation. In studies using hematopoietic cell lines expressing different combinations of IRS-proteins, we found that independently of the IRS-protein expressed (IRS-1 or

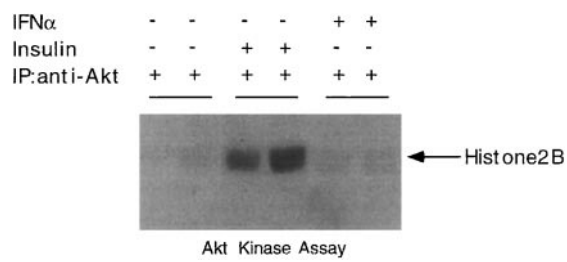

FIG. 6. Activation of the Akt kinase by insulin but not IFNα in U-266 cells. Cells were serum starved for 2 hours and they were subsequently treated with either 100 nM insulin or 10^4 U/ml IFNα as indicated. Duplicate samples were immunoprecipitated with an anti-Akt antibody and subjected to an Akt kinase assay using exogenous histone 2B as a substrate. The proteins were analyzed by SDS-PAGE and the phosphorylated form of histone 2B was detected by autoradiography.

IRS-2), p85 preferentially associates with IRS-proteins as compared to Stat-3. These findings indicate that the IRS-cascade is the major pathway regulating engagement of the PI 3′-kinase in interferon signaling. Our data also demonstrate that a receptor completely devoided of tyrosine phosphorylation sites can sustain activation of the serine kinase activity of the PI 3′-kinase, establishing that tyrosine phosphorylation of the Type I IFN receptor is not required for such an activation.

The physiologic relevance of activation of the PI 3′-kinase in interferon signaling remains to be established in further studies. However, our data that the Akt kinase is not activated during engagement of the Type I IFN receptor, strongly suggest that there is a differential activation of elements downstream of the PI 3′-kinase in response to IFN- versus growth factor-induced activation. Such differential function of the PI 3′-kinase may account for signaling specificity in response to different ligands.

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