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Interaction of p59^{fyn} with Interferon-Activated Jak Kinases

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During IFNα stimulation, p59^{fyn} associates with the Type I IFNR-associated Tyk-2 kinase in several human hematopoietic cell lines *in vivo*. This interaction is direct, and is mediated by the SH2 domain in p59^{fyn}, as shown by binding studies using glutathione-S-tranferase fusion proteins and far western blots. Furthermore, in response to IFNα-treatment of cells, the SH2 domain of Fyn interacts with the Tyk-2-associated *c-cbl* proto-oncogene product. In a similar manner, during IFNγ stimulation, p59^{fyn} associates via its SH2 domain with the activated form of the IFNγ-dependent Jak-2 kinase. These data suggest that p59^{fyn} is a common element in IFNα and IFNγ signaling, and is selectively engaged by the Type I or II IFN receptors via specific interactions with dis-tinct Jak kinases. © 1997 Academic Press

Interferons (IFNs) are pleiotropic cytokines that exert antiproliferative, antiviral and immunomodulatory activities on normal and neoplastic cells (1). Although the precise mechanisms by which these cytokines exert their biological effects remain unknown, significant advances have been recently made in our understanding of the signaling events that take place during binding of interferons to their receptors. Binding of IFNα to the Type I IFN receptor results in activation of the Janus family Tyk-2 and Jak-1 kinases (2-6), and tyrosine phosphorylation of various signaling elements, including the α and β subunits of the Type I IFNR (7-9), Stat-proteins (10-13), IRS-proteins (14,15), and the Vav (16) and C-cbl (17) proto-oncogenes. Furthermore, engagement of Jak kinases during IFNα stimulation appears to regulate activation of the lipid (14) and serine (18) kinase activities of the phosphatidylinositol 3′-kinase. On the other hand, binding of IFNγ to the Type II IFN receptor results in activation of the Jak-2 and Jak-1 kinases (3,4,19) and tyrosine phosphorylation and homodimerization of Stat-1 (reviewed in 13).

Although the involvement of Jak kinases in interferon signaling is well documented, the role of kinases of other families is unknown. In the present study we sought to determine whether the Src-related tyrosine kinase Fyn is engaged in interferon-signaling. This kinase has been previously implicated in signal transduction by various receptors, including the PDGF, GSF-1, and B and T cell-antigen receptors (20-23). Our data demonstrate that p59^{fyn} interacts via its SH2 domain with the activated forms of Tyk-2 or Jak-2, in response to IFNα or IFNγ stimulation respectively, suggesting that this kinase may be involved in the regulation of interferon-signaling pathways downstream of Jaks.

EXPERIMENTAL PROCEDURES

Cells and reagents. The Molt-16 (acute T-cell lymphocytic leukemia), Molt-4 (acute T-cell lymphocytic leukemia), Daudi (lymphoblastoid), and U-266 (multiple myeloma) 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. Human recombinant IFNα2 was provided by Hoffmann Laroche. Human recombinant IFNcon1 (IFNα) was provided by Amgen Inc. Human recombinant IFNγ was provided by Genentech Inc. The antiphosphotyrosine monoclonal antibody (4G-10) and the anti-Jak-2 polyclonal antibody were obtained from Upstate Biotechnology (Lake Placid, NY). The polyclonal anti-Tyk-2 antibody has been raised against a synthetic peptide corresponding to the C-terminal 15 aminoacids of Tyk-2 (8,24). The monoclonal antibody against Tyk-2 was obtained from Transduction Laboratories (Lexin-gton, KY) and was used for immunoblotting. The polyclonal anti-body against the tyrosine kinase Fyn has been described elsewhere (25). A polyclonal antibody against C-cbl was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

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Abbreviations: ISGs, interferon-stimulated genes; PAGE, polyacrylamide gel electrophoresis; IFNR, interferon receptor; Stat, signal transducer and activator of transcription.

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Immunoprecipitations and immunoblotting. Cells were stimulated with the indicated IFNs (10^4 U/ml) for the indicated time periods. In some experiments the cells were serum starved by incubation in serum-free DMEM (Life Technologies Inc.) for 2 hours prior to interferon stimulation. After stimulation, the cells were rapidly centrifuged and lysed as previously described (26). In some experiments the cell lysates were pre-cleared with nonimmune rabbit serum prior to immunoprecipitation. Immunopreci-pitations and immunobloting were performed as previously descri-bed (26).

Preparation of glutathione-S-transferase fusion proteins and binding studies. The generation of the pGEX-FynSH2 and pGEX-FynSH3 constructs has been described elsewhere (25). Production of glutathione-S-transferase fusion proteins and binding experiments using lysates from cells stimulated with 10^4 U/ml of IFNα or IFNγ were performed as previously described (14,27).

Far western blots. Cells were treated in the presence or absence of IFNα as indicated, cell lysates were immunoprecipitated with the indicated antibodies, analyzed by SDS-PAGE and transferred to PVDF membranes as described in the procedure for immunoblotting. The membranes were subsequently incubated for 1 hr at room temperature with approximately 2-4 μg of the FynSH2 fusion protein in 10 ml TBST-0.5% BSA, washed extensively with TBST, and then incubated with a polyclonal anti-GST antibody (Santa Cruz Biotechnology), prior to developing by the ECL method.

RESULTS AND DISCUSSION

We initially performed experiments in which various IFNα-sensitive cell lines were treated with IFNα, and after cell lysis the lysates were immunoprecipitated with an anti-p59^{fyn} antibody and immunoblotted with antiphosphotyrosine. In Molt-16 cells, a 59 kD protein corresponding to p59^{fyn} was detectable prior to and after IFNα stimulation (Fig. 1A). Under these conditions no IFNα-dependent increase in the phosphorylation of p59^{fyn} was noticeable. However, on longer exposure of the blots, a 135 kD tyrosine phosphorylated protein was clearly detectable in anti-p59^{fyn} immunoprecipitates from IFNα-stimulated cells (Fig. 1B). Similar results were obtained in experiments using Daudi (Fig. 1C) and Molt-4[2] cells. As this protein co-migrated with the IFNα-phosphorylated form of Tyk-2, we sought to determine whether it corresponds to Tyk-2. αTyk-2 immunoblots demonstrated that Tyk-2 was present in anti-p59^{fyn} immunoprecipitates from lysates of IFNα stimulated cells (Fig. 1D and 1E), confirming that the 135 kD phosphoprotein is indeed Tyk-2.

As the association of p59^{fyn} with Tyk-2 was IFNα-dependent, we sought to determine whether it involves binding of the SH2 domain of Fyn to Tyk-2. Cells were treated with IFNα and cell lysates were bound to a GST-FynSH2 fusion protein prior to SDS-PAGE analysis and immunoblotting with antiphosphotyrosine or anti-Tyk-2. Fig. 2A and 2B show that the Fyn-SH2 fusion protein binds to the phosphorylated form of Tyk-2 *in vitro,* strongly suggesting that the p59^{fyn}-Tyk-2 interaction is mediated by the FynSH2 domain. The IFNα-induced association of FynSH2-phosphorylated

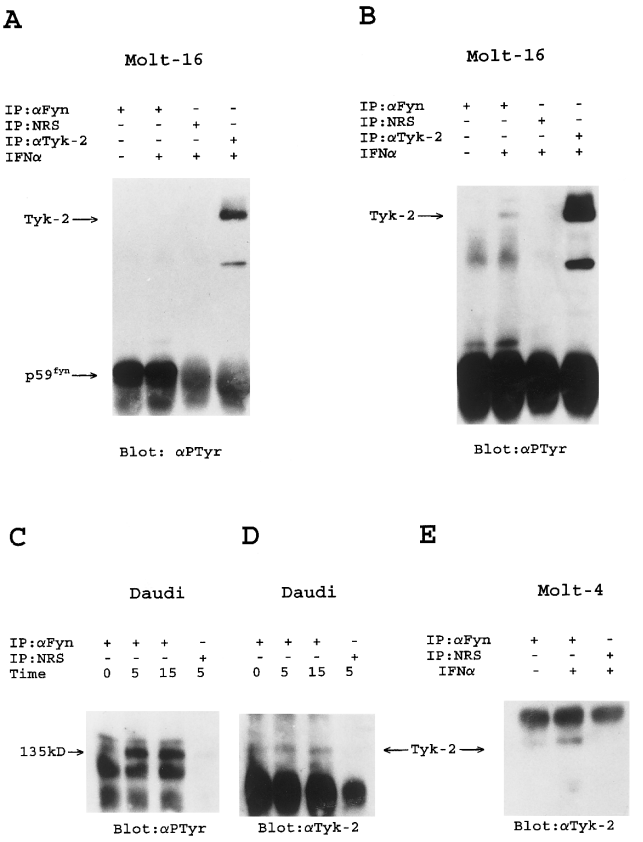

FIG. 1. IFNα-dependent association of p59^{fyn} with Tyk-2. **A.** Antiphosphotyrosine immunoblot. Serum starved Molt-16 cells (1.2×10^7/ml) were treated with IFNα for 5 min at 37°C as indicated, cell lysates were pre-cleared with non-immune rabbit serum (NRS), and were subsequently immunoprecipitated with anti-p59^{fyn} or NRS or αTyk-2 as indicated. **B.** Longer exposure of the blot shown in A. **C.** Antiphosphotyrosine immunoblot. Serum starved Daudi cells (1.7×10^7/ml) were treated with IFNα for the indicated times at 37°C, cell lysates were pre-cleared with NRS, and were subsequently immunoprecipitated with anti-p59^{fyn} or NRS as indicated. **D.** Anti-Tyk-2 immunoblot. The blot shown in C was stripped and reblotted with an antibody against Tyk-2. **E.** Anti-Tyk-2 immuno-blot. Molt-4 cells (3.7×10^7/lane) were treated with IFNα for 5 min at 37°C as indicated, cell lysates were pre-cleared with NRS and immunoprecipitated with the indicated antibodies.

Tyk-2 occured within 5 min of treatment of cells with IFNα, was still detectable after 15 min, and diminished after 30-90 min of IFNα treatment of cells (Fig. 2C), suggesting that such associa-tion is rapid and transient. To determine whether the SH2 domain in Fyn interacts directly with tyrosine phosphorylated Tyk-2, we performed far western blots on anti-Tyk-2 immunoprecipitates from Daudi or Molt-4 cell lysates. Fig. 3 shows that the GST-FynSH2 fusion protein bound to the IFNα-phosphorylated form of Tyk-2, strongly suggesting that the FynSH2-Tyk-2 interaction is direct, without requirement for adaptor proteins.

We have previously shown that the C-cbl proto-oncogene product is constitutively associated with the Tyk-

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

2 tyrosine kinase, and is tyrosine phosphorylated during IFNα stimulation (17). This protein has been also previously shown to interact with the SH3 domains of src kinases (Fyn, Lyn, Lck, and Src) (28-30), and with the SH2 domains of Fyn and other src kinases during activation of the T-cell antigen receptor (28). In addition, C-cbl interacts with the phosphatidylinositol 3′-kinase in T- and B-cells (31-33). We sought to determine whether the SH3 and SH2 domains of p59^{fyn} also interact with C-cbl during IFNα stimula-tion. Consistently with previous reports (28-30), a GST-FynSH3 fusion protein bound to C-cbl from lysates of untreated cells (Fig. 4A). Treatment of the cells with IFNα did not result in further increase in the binding of C-cbl to the Fyn-SH3 domain (Fig. 4A). A GST-FynSH2 fusion protein, however, bound to C-cbl in an IFNα-dependent manner (Fig. 4B). Thus, in addition to Tyk-2, it is likely that p59^{fyn} interacts via its SH2 domain with the IFNα-phosphorylated form of C-cbl. Previous studies have also shown that interaction of p85 regulatory subunit of the PI 3′-kinase with the SH3 domain of p59^{fyn} regulates the catalytic activity of the PI 3′-kinase (34). We therefore sought to determine the interaction of the SH3 domain with p85 from lysates of IFNα-treated cells. Fig. 4C shows that the SH3 domain of p59^{fyn} bound to p85 prior to and after IFNα stimulation, consistent with a constitutive interaction of this motif in p59^{fyn} with p85, and in agreement with previous reports. On the other hand, as expected, no interaction of the SH2 domain of p59^{fyn} with p85 was detectable (Fig. 4C). Thus, interaction of p59^{fyn} with Tyk-2 and C-cbl during IFNα stimulation, may provide a pathway

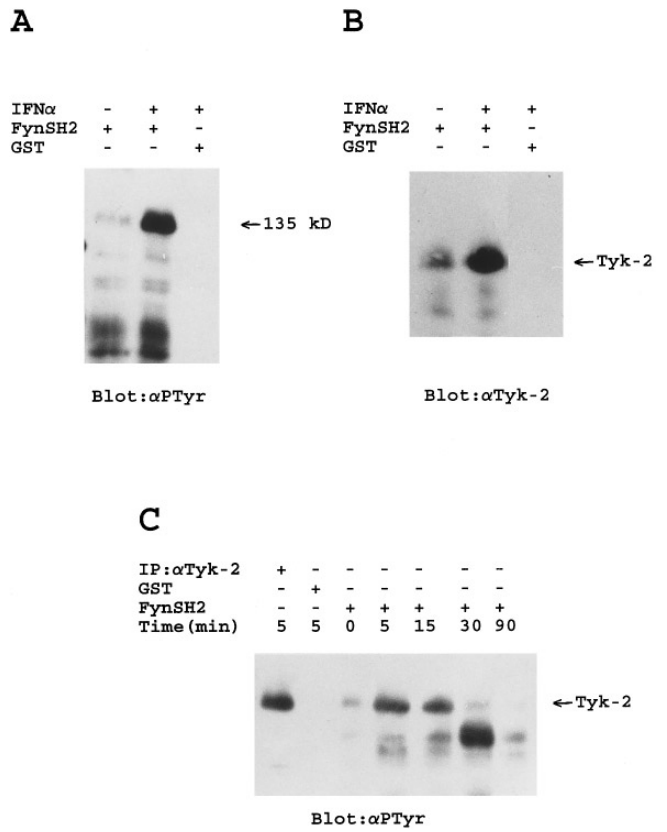

FIG. 2. Binding of the SH2 domain of p59^{fyn} to activated Tyk-2. **A.** Antiphosphotyrosine immunoblot. Molt-4 cells (1.5×10^8/ lane) were incubated for 5 min at 37° C in the presence or absence of IFNα as indicated, cell lysates were bound to either GST alone or a GST-FynSH2 fusion protein as indicated, and bound proteins were analyzed by SDS-PAGE. **B.** Anti-Tyk-2 immunoblot. Daudi cells (5×10^7/lane) were incubated for 5 min at 37°C in the presence or absence of IFNα as indicated, cell lysates were bound to either GST alone or a GST-FynSH2 fusion protein as indicated, and bound proteins were analyzed by SDS-PAGE. **C.** Molt-4 cells (3.5×10^7/lane) were treated with IFNα for the indicated times at 37°C, and cell lysates were either immunoprecipitated with an anti-Tyk-2 antibody, or bound to either GST alone or a GST-FynSH2 fusion protein as indicated. Proteins were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine.

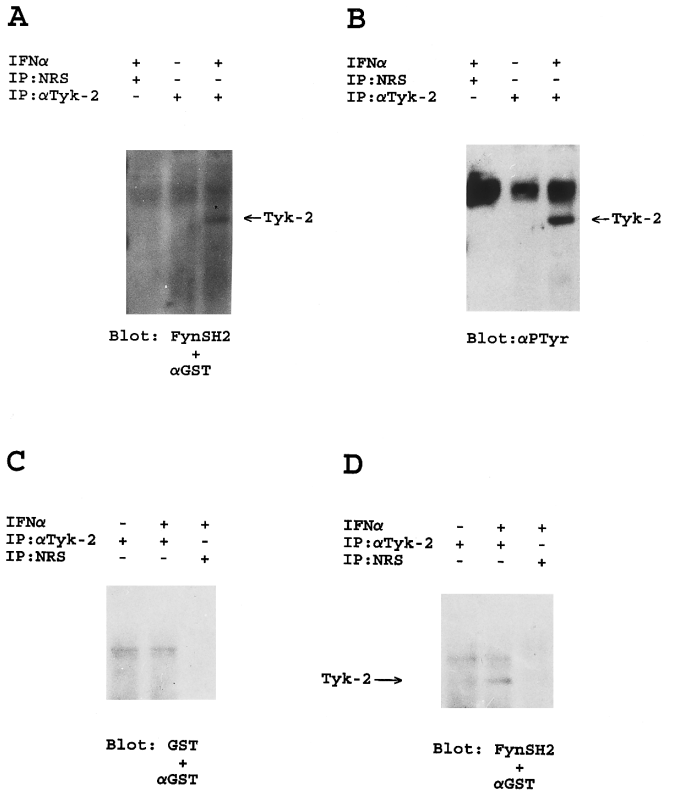

FIG. 3. Binding of the FynSH2 domain to Tyk-2 *in vitro*. **A.** Daudi cells were incubated for 5 min at 37°C in the presence or absence of IFNα as indicated. Cell lysates were immunoprecipitated with either αTyk-2 or NRS as indicated, and proteins were analyzed by SDS-PAGE and transferred to PVDF. A FynSH2 far western blot was subsequently performed as indicated in the methods section. **B.** The blot shown in A was stripped and re-blotted with an antiphosphotyrosine monoclonal antibody. **C.** and **D.** Molt-4 cells were incubated for 10 min at 37° C in the presence or abscence of IFNα as indicated. Equal amounts of cell lysates were used for immunoprecipitations with the indicated antibodies for the C or D panel. After SDS-PAGE analysis, far western blots were performed with either GST alone **(C)** or the FynSH2 fusion protein **(D)**.

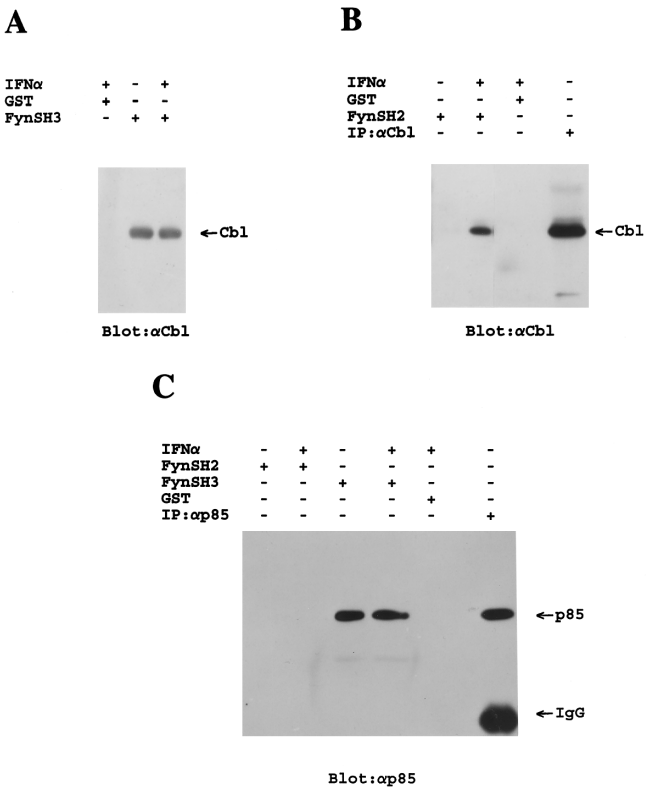

FIG. 4. Interaction of the SH3 and SH2 domains of p59^{fyn} with IFNα-signaling elements during IFNα treatment of cells. **A.** Molt-16 cells (3.7×10^7/lane) were treated in the presence or absence of IFNα for 5 min as indicated, cell lysates were bound to either GST alone or a GST-FynSH3 fusion protein as indicated, and bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against C-cbl. **B.** Molt-4 cells (4.1×10^7/lane) were treated in the presence or absence of IFNα for 5 min as indicated, cell lysates were either immunoprecipitated with an antibody against C-cbl as indicated, or were bound to either GST alone or a GST-FynSH2 fusion protein as indicated, and bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against C-cbl. **C.** KG1 cells were serum starved for 3 hours in serum-free RPMI, and were subsequently incubated in the presence or absence of IFNα for 5 min as indicated. Cell lysates were bound to either GST alone or the indicated fusion proteins, and bound proteins were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody against p85α.

for the regulation PI 3′-kinase, which has been previously shown to be activated in response to IFNα treatment of cells (14,18).

We subsequently determined whether p59^{fyn} associates with Jak-2, which is activated during IFNγ stimulation (3,4,17). Lysates from IFNγ treated U-266 human myeloma cells were immuno-precipitated with the anti-p59^{fyn} antibody and immunoblotted with antiphosphotyrosine. A 130 kD phosphoprotein that co-migrated with Jak-2 was clearly detectable in the anti-p59^{fyn} immunopreci-pitates from IFNγ stimulated cells, suggesting that p59^{fyn} interacts with Jak-2 in an IFNγ-dependent manner(Fig. 5A). Furthermore, GST fusion protein binding experiments demonstrated that during IFNγ stimulation the SH2 domain of Fyn binds to the phosphorylated form of Jak-2 (Fig. 5B and 5C). Thus, IFNγ stimulates the association of p59^{fyn} with Jak-2, and such an interaction most likely requires the SH2 domain in Fyn.

The discovery of the Jak-Stat pathways reveals a cellular mechanism by which Type I and II IFN- generated signals are transmitted directly from the cell surface to the nucleus to regulate gene transcription (13). Although the involvement of Jak kinases in interferon-signaling is well documented, the role that kinases of other families may play in the generation of interferon signals is not known. p59^{fyn} is a member of the Src family of kinases. The protein contains SH2 and SH3 domains, both of which are essential for regulation of its enzymatic activity (35-37). The importance of this kinase during signal transduction by tyrosine kinase receptors has been established in various systems. The PDGF and CSF-1 receptors associate with p59^{fyn} during binding of their respective ligands (20,21). Inhibition of Fyn function by injection of anti-Fyn antibodies

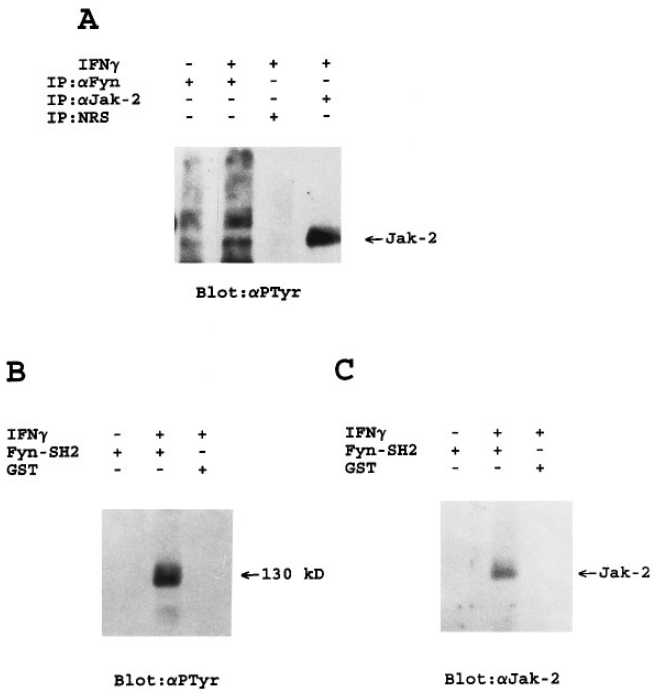

FIG. 5. p59^{fyn} associates via its SH2 domain with Jak-2 during IFNγ stimulation. **A.** U-266 cells (1.8×10^7/lane) were treated with IFNγ for 5 min at 37°C as indicated, cell lysates were immunoprecipitated with the indicated antibodies or non-immune rabbit serum (NRS). Proteins were analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. **B.** Molt-4 cells (1.3×10^8/ lane) were treated for 3 min at 37°C as indicated, and cell lysates were bound to a GST-FynSH2 fusion protein or GST alone as indicated. Bound proteins were analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. **C.** The blot shown in **B** was stripped and re-blotted with with an anti-Jak-2 antibody.

or DNA encoding dominant-negative proteins, reduces the PDGF mitogenic response, underscoring the importance of $p59^{fyn}$ in the generation of PDGF signals (38). In addition, $p59^{fyn}$ associates with the B- and T- cell antigen receptors, and participates in signaling by these receptors during antigen stimulation (23,24).

In the current report we present the first evidence that Fyn, a Src-kinase, interacts with Jak kinases, and may be a component of interferon-signaling pathways downstream of Jaks. As multiple signaling proteins are engaged during interferon-stimulation, it is possible that some of them are targets for the activity of $p59^{fyn}$, while others act as direct substrates for Jak kinases. Engagement of $p59^{fyn}$ in IFNα signaling may provide a pathway for activation of the PI 3′-kinase, as the p85 subunit of the PI 3′-kinase interacts with the SH3 domain of $p59^{fyn}$ and with C-cbl, that also associates with $p59^{fyn}$.

Such a model for a function of $p59^{fyn}$ (and possibly other Src-kinases) downstream of Jaks, is also consistent with the pre-viously reported interaction of members of the Syk-Zap-70 family of kinases with Src-kinases. Previous studies have established that the SH2 domains of Src family kinases interact with the tyrosine kinase Syk during B-cell activation (39), while the SH2 domain of $p56^{lck}$ interacts with Zap-70 during anti-CD3 activation (40). It has been also suggested that Syk acts as an immediate receptor-activated kinase upstream of Zap-70, $p56^{lck}$, and $p59^{fyn}$ in T-cells (41). Our finding, that $p59^{fyn}$ interacts with interferon-dependent Janus kinases, suggests the existence of a similar mechanism involving co-operation between Src kinases and Jaks during generation of interferon signals. Such a concept is further supported by a recent study that demonstrated that transformation of cells by the Src-oncoprotein induces tyrosine phosphorylation and DNA-binding activity of Stat-3 (42), as well as by a recent report that established that $p60^{src}$ and $p59^{fyn}$ can interact with Stat-1 and regulate Jak-independent activation of Stats in cells lacking Jaks during treatment with phosphatase inhibitors (43). Our data complement such findings and strongly suggest that Jaks regulate engagement of $p59^{fyn}$ in interferon signaling. In addition, they raise the possibility that additional members of the Src-family of kinases are engaged in signaling for interferons and other cytokines by similar mechanisms. Indeed, we have recently observed that $p56^{lyn}$ also associates with Tyk-2 in an IFNα-dependent manner[2] in cells of B-cell origin, and studies are currently underway to determine the precise mechanism of this interaction.

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