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The SH2 Domain-containing Tyrosine Phosphatase PTP1D Is Required for Interferon α/β-induced Gene Expression*

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Interferons (IFNs) induce early response genes by stimulating Janus family (Jak) tyrosine kinases, leading to tyrosine phosphorylation of Stat (signal transducer and activator of transcription) proteins. Previous studies demonstrated that a protein-tyrosine phosphatase (PTP) is required for activation of the ISGF3 transcription complex by IFNα/β, but the specific PTP responsible remained unidentified. We now show that the SH2 domain containing tyrosine phosphatase PTP1D (also designated as SHPTP2, SHPTP3, PTP2C, or Syp) is constitutively associated with the IFNα/β receptor and becomes tyrosine-phosphorylated in response to ligand. Furthermore, transient expression of a phosphatase-inactive mutant or the COOH-terminal SH2 domain of PTP1D causes a dominant negative effect on IFNα/β-induced early response gene expression. These results provide strong evidence that PTP1D functions as a positive regulator of the IFNα/β-induced Jak/Stat signal transduction pathway.

Interferons as well as many other cytokines and growth factors stimulate the expression of early response genes by inducing the tyrosine phosphorylation of SH2-containing transcription factors termed Stats (1–5), a process that involves the activation and tyrosine phosphorylation of members of the Janus family of protein-tyrosine kinases (Jaks) (6–9). Tyrosine phosphorylation of the Stat proteins causes them to either homo- or heterodimerize and to translocate to the nucleus, where they interact with enhancer elements in a variety of promoters to initiate transcription (10, 11). Serine phosphorylation of Stat1 by mitogen-activated protein kinase (MAPK, ERK2) is required in addition to tyrosine phosphorylation for maximum transcriptional activation (12, 13).

Several components of the IFNα/β[1] signaling pathway have been identified and molecularly cloned, such as the two subunits (α and β) of the IFNα/β receptor (14–16). Binding of IFNα/β to its receptor causes the rapid activation of the Jak tyrosine kinases Jak1 and Tyk2 (6, 7) which results in tyrosine phosphorylation of both Stat1α (p91) and Stat2 (p113) (1, 17) and the formation of at least two transcription factor complexes. One complex, composed of a heterotrimer of Stat1α, Stat2, and the DNA binding component p48 (ISGF3γ), binds to interferon-stimulated response elements (ISREs) (18, 19), whereas Stat1α homodimers bind to γ response region (GRR) elements (20).

Although much attention has been devoted to the tyrosine kinases involved in Stat activation, the role of tyrosine phosphatases in IFNα/β signal transduction is less understood. Evidence from both *in vitro* and *in vivo* studies indicated that tyrosine phosphatases can act as positive as well as negative regulators of the IFNα/β-induced Jak/Stat pathway (17, 21, 22). Recently, we were able to demonstrate that the SH2 domain containing tyrosine phosphatase PTP1C (SHPTP1, HCP, or SHP) functions as a suppressor of IFNα/β signal transduction in hematopoietic cells by specific down-modulation of Jak1 tyrosine phosphorylation (23). Studies of Stat activation by IFNα/β in cell homogenates provided evidence that a tyrosine phosphatase is also required to initiate signal transduction (17). IFNα/β-induced formation of ISGF3 could be inhibited if the tyrosine phosphatase inhibitor orthovanadate was added to a cell membrane fraction prior to IFNα/β. However, if vanadate was added a few minutes after IFNβ, then no inhibition of ISGF3 formation was observed. In contrast, the tyrosine kinase inhibitor genistein was able to abolish Stat activation when added either before or after IFN. These results suggested that a tyrosine phosphatase activity was required to initiate the signaling cascade but not to maintain its activity. Several reports have implicated the SH2 domain containing tyrosine phosphatase PTP1D as a positive (signal-enhancing) mediator of growth factor signaling (24–27). PTP1D, also known as SHPTP2, PTP2C, and Syp, is ubiquitously expressed as is the homologue in *Drosophila*, *csw* (28). Genetic analysis indicates that Csw is required for signaling by *Torso* (29). Likewise, vertebrate PTP1D has been implicated in positive signaling by the insulin receptor (26), the epidermal growth factor receptor (30) and the fibroblast growth factor receptor (25). PTP1D is tyrosine-phosphorylated upon stimulation of appropriate cells with the growth factors (31), an event that has been shown to lead to the activation of the enzyme (32, 33). We therefore wanted to explore the possible involvement of PTP1D in the regulation of the IFNα/β-induced Jak/Stat pathway.

MATERIALS AND METHODS

Cells—U266 cells were grown as a suspension culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (UBI). Primary human diploid fibroblasts were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal calf serum.

Whole Cell Extracts—After treatment, cells were diluted with ice-

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[1] The abbreviations used are: IFNs, interferons; PTP, protein-tyrosine phosphatase; ISRE, interferon-stimulated response element; GRR, IFNγ response element; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CMV, cytomegalovirus; α-subunit of IFNα/β receptor, the cDNA clone of the human IFNα/β receptor described by Uze *et al.* (16).

cold phosphate-buffered saline and centrifuged at 1500 × *g* for 10 min at 4 °C, washed with PBS, and resuspended in 1 ml of lysis buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (all from Sigma). For some experiments, 1% digitonin was substituted for Triton X-100. The lysate was vortexed, incubated on ice for 10 min, and centrifuged at 18,000 × *g* for 10 min at 4 °C.

Precipitation with Antibodies or GST Fusion Proteins—Lysates were incubated with the indicated antibodies for 2 h prior to the addition of Protein G-Sepharose beads (Pharmacia Biotech Inc.) and incubation for an additional hour. All antibodies used for immunoprecipitation have been described (12). GST fusion proteins representing the cytoplasmic domain of the IFNα/β receptor (34) bound to agarose beads were incubated with the extracts at 4 °C for 2–12 h. In either case, the beads were pelleted at 15,000 × *g* for 2 min and washed three times with ice-cold lysis buffer (1 ml) (12).

Western Blotting—Proteins were separated on 4–16% SDS-PAGE gels (Novex) and transferred to Immobilon (Millipore). Membranes were probed with monoclonal antibodies against PTP1D (Transduction Laboratories) or the α-chain of the IFNα/β receptor (generously provided by Susan Goelz and Christopher Benjamin) or phosphotyrosine (PY20, ICN) using concentrations and conditions recommended by the manufacturer. Immunoblots were developed using appropriate secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

Plasmids and Transfections—Phagemid pBluescript SK-PTP1D was obtained by screening a λZAP human fetal brain cDNA library. Single-stranded DNA of pBluescript SK-PTP1D was made from an *Escherichia coli* strain CJ236 and used for site-directed mutagenesis (Muta-Gene *In Vitro* Mutagenesis Kit, Bio-Rad). The *Bam*HI and *Hin*dIII fragments encoding human PTP1D and PTP1D (Cys^{459} → Ser) were cloned into a eukaryotic expression vector pCMV5 digested with *Bgl*II and *Hin*dIII to generate pCMV5-PTP1D and pCMV5-PTP1D (Cys → Ser), respectively. The polymerase chain reaction (PCR) was used to construct pCMV5-PTP1D (N-SH2) and pCMV5-PTP1D (C-SH2). The PCR primers for PTP1D (N-SH2) were 5′-AACTGGATCCATGACATCGCGGAGATG-GTTTC and 5′-GTCAGAATTCTTACC-TTTCAGAGGTAGG. The PCR primers for PTP1D (C-SH2) were 5′-AACT-GGATCCGCAGATCCTAC-CTCTG and 5′-GTCGGAATTCTTATTTGACTTT-ATCTGTGGTC. The underlined *Bam*HI and *Eco*RI sites were used for cloning the PCR product. The *Eco*RI fragments encoding PTP1C and PTP1C (Cys → Ser) were cloned into the pCMV5 *Eco*RI site to generate pCMV5-PTP1C and pCMV5-PTP1C (Cys → Ser), respectively.

Primary human fibroblasts were transfected as described with an ISRE derived from the interferon-stimulated responsive gene ISG15 linked to a thymidine kinase minimal promoter-luciferase reporter. In addition to the reporter construct, plasmids containing cDNAs corresponding to either a phosphatase-inactive PTP1C or PTP1D or the NH_2- or COOH-terminal SH2 domains of PTP1D were cotransfected. These plasmids all contained a cytomegalovirus (CMV) promoter. To normalize for DNA in the transfection, a CMV-driven β-galactosidase cDNA was included when the PTP1D plasmids were not present. Twenty hours after transfection, IFNβ (10^3 units/ml) was added to the cells for 6 h before preparation of cell lysates and assay of luciferase activity.

RESULTS AND DISCUSSION

We had previously noted that the initiation of Stat activation by either IFNα/β or IFNγ in an *in vitro* signaling system requires the catalytic activity of a tyrosine phosphatase (17, 21). The SH2 domain containing tyrosine phosphatase PTP1D has been implicated as a positive regulator of mitogenic responses in many growth factor signal transduction pathways (24, 25), but its involvement in cytokine receptor signaling had remained unclear. We therefore wanted to investigate whether the tyrosine phosphatase required for signal transduction through Stat proteins was PTP1D.

In order to determine whether PTP1D would associate with the IFNα/β receptor, affinity precipitations were performed using lysates derived from untreated or IFNβ-treated U266 cells. GST-fusion proteins containing either the entire 100-amino acid cytoplasmic domain of the α-subunit of the IFNα/β receptor, or the 50-amino acid membrane-proximal or the 50-amino acid carboxyl-terminal region were bound to glutathione agarose and incubated with the cell lysates for 2–12 h. Bound proteins were then separated by SDS-PAGE, transferred to Immobilon, and the membrane was probed with a monoclonal antibody against PTP1D. PTP1D was found to associate with GST fusion proteins representing either the entire cytoplasmic domain of the IFNα/β receptor or the membrane-proximal 50-amino acid region (Fig. 1*A*, *lanes 1*, *2*, *5*, and *6*), but did not bind to the construct containing the carboxyl-terminal 50 amino acids (*lanes 3* and *4*). Interestingly, the binding of PTP1D to the GST fusion proteins occurred whether or not cells were incubated with IFNβ prior to preparation of the lysates. To confirm the association of PTP1D with the IFNα/β receptor, coimmunoprecipitation experiments were performed using a mono-

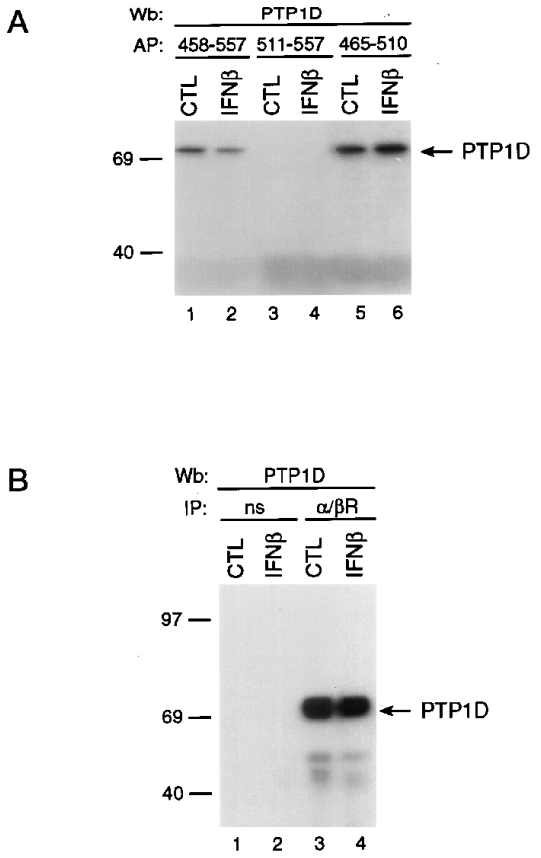

FIG. 1. **Interaction of proteins with the cytoplasmic domain of the α subunit of the IFNα/β receptor.** U266 cells (1–2 × 10^6 cells/sample) were incubated for 5 min at 37 °C with or without recombinant human IFNβ (10^3 units/ml). Cells were diluted with ice-cold PBS and centrifuged at 1500 × *g* for 10 min at 4 °C, washed with PBS, and resuspended in lysis buffer containing 20 mM Hepes (pH 7.4), 1 mM vanadate, 10 mM β-glycerophosphate, 100 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride. The lysate was vortexed, incubated on ice for 10 min, and centrifuged at 18,000 × *g* for 10 min at 4 °C. *A*, GST fusions containing either the entire coding region (458–557) of the cytoplasmic domain of the α subunit of the IFNα/β receptor (*lanes 1* and *2*), the COOH-terminal 50 amino acids (511–557) (*lanes 3* and *4*), or the membrane-proximal 50 amino acids of the cytoplasmic tail (465–510) (*lanes 5* and *6*) were bound to the beads and incubated with the extracts at 4 °C for 2 to 12 h. The beads were subsequently sedimented at 15,000 × *g* for 2 min and washed three times with ice-cold lysis buffer (1 ml). Proteins bound to the beads were separated using SDS-PAGE (4–16% gels) and transferred to Immobilon. The membranes were then incubated with monoclonal antibodies to PTP1D (Transduction Laboratories). *Wb*, Western blotting antibody used to probe the membrane; *AP*, affinity precipitation (the GST fusion protein with which the cellular extracts were incubated). *B*, U266 cells were incubated without or with IFNβ (10^3 units/ml) for 5 min. Cellular extracts were prepared and proteins were immunoprecipitated either with control antibodies (*lanes 1* and *2*) or with monoclonal antibodies to the extracellular domain of the IFNα/β receptor (*lanes 3* and *4*). The immunoprecipitates (*IP*) were resolved by SDS-PAGE, the proteins were transferred to Immobilon, and the blot was probed (*Wb*) with a monoclonal antibody to PTP1D.

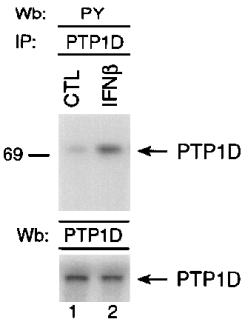

FIG. 2. **IFNβ stimulates the tyrosine phosphorylation of PTP1D.** U266 cells were incubated without (*lane 1*) or with 10^3 units/ml recombinant human IFNβ (*lane 2*) for 10 min at 37 °C. PTP1D was immunoprecipitated from the lysates with a polyclonal antibody generated against a peptide corresponding to the 17 carboxyl-terminal amino acids of human PTP1D. The immunoprecipitates were resolved by SDS-PAGE, the proteins were transferred to Immobilon, and the blot was probed with a monoclonal antibody to phosphotyrosine (4G10, UBI). To ensure that equal amounts of protein were present in the immunoprecipitates, a separate aliquot was probed directly for PTP1D (*lower panel*).

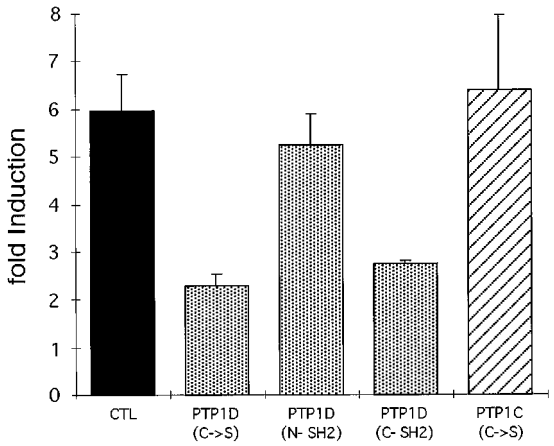

FIG. 3. **Inhibition of IFNβ-stimulated transcription of an ISRE reporter plasmid in cells expressing either dominant negative PTP1D or the SH2 domains of PTP1D.** Primary human fibroblasts were transfected as described with an ISRE derived from the interferon-stimulated responsive gene ISG15 linked to a thymidine kinase minimal promoter-luciferase reporter. In addition to the reporter construct, plasmids containing cDNAs corresponding to either catalytically inactive PTP1D (*PTP1D (C→S)*), the amino-terminal SH2 domain (*PTP1D (N-SH2)*), or the carboxyl-terminal SH2 domain (*PTP1D (C-SH2)*) of PTP1D were cotransfected. As a control for specificity, a plasmid encoding a catalytically inactive form of PTP1C (*PTP1C (C→S)*) was also included. These plasmids all contained a CMV promoter. To normalize for DNA in the transfection, a CMV-driven β-galactosidase cDNA was included when no PTP plasmids were present (*CTL*). Twenty hours after transfection, IFNβ (10^3 units/ml) was added to the cells for 6 h before preparation of cell lysates and assay of luciferase activity. The reporter construct without the ISRE showed no increased luciferase activity in the presence of IFNβ (data not shown). The standard deviations for these experiments (S.D.) are denoted by *error bars*.

clonal antibody directed against the α-subunit of the receptor. As observed in the affinity precipitations with the GST fusion proteins, PTP1D associated independently of IFN stimulation with the IFNα/β receptor (Fig. 1).

PTP1D has been shown to undergo tyrosine phosphorylation in response to treatment of cells with various growth factors, which correlates with an increase in its enzymatic activity (32, 33). To investigate whether treatment of cells with IFN would also result in the tyrosine phosphorylation of PTP1D, we performed antiphosphotyrosine blots on immunoprecipitated PTP1D. As shown in Fig. 2, although a basal tyrosine phosphorylation of PTP1D was detected, this phosphorylation increased as a consequence of IFN stimulation (*lane 1 versus 2*).

The fact that an association between PTP1D and the IFNα/β receptor was detected, and it became tyrosine-phosphorylated as a consequence of IFNα/β treatment, suggested that the phosphatase might regulate the activation of the Jak/Stat pathway. To examine the role of PTP1D in IFN induction of early response genes requiring activation of the Jak-Stat pathway, we used transient transfection assays to determine whether expression of catalytically inactive, dominant negative PTP1D affected IFNβ activation of a luciferase reporter containing the interferon-stimulated response element (ISRE) (Fig. 3). These experiments were done with primary human fibroblasts since U266 cells could not be efficiently transfected. Fibroblasts were either transfected with the luciferase construct alone or with plasmids encoding either a PTP1D with a cysteine to serine mutation in the catalytic domain rendering the protein phosphatase-inactive, or the NH_2-terminal or the COOH-terminal SH2 domain of PTP1D. A vector corresponding to PTP1C with the same cysteine to serine mutation as in PTP1D was also used as a control for specificity. After transfection, fibroblasts were incubated with or without IFNβ for 5 h, the cells were lysed, and luciferase activity was assayed. IFNβ consistently stimulated a 6- to 7-fold increase in luciferase activity in cells transfected with the ISRE-containing luciferase reporter. Expression of the phosphatase-inactive PTP1D as well as the COOH-terminal SH2-domain resulted in a 50–60% decrease in IFNβ-induced luciferase activity, whereas expression of the NH_2-terminal SH2 domain or a dominant negative PTP1C was without effect. Similar results were obtained when a luciferase reporter construct with a GRR enhancer was used (data not shown). Thus, it appears that PTP1D can function as a positive regulator of IFNα/β stimulation of early response genes that require activation of the Stat transcription factors.

In summary, our results indicate that induction by IFNα/β of early response genes through Stat binding enhancers requires the catalytic activity of PTP1D. Furthermore, we demonstrated that this SH2 domain containing tyrosine phosphatase is constitutively associated with the IFNα/β receptor and undergoes tyrosine phosphorylation in response to ligand binding. PTP1D has been established as a positive regulator of several growth factor-activated signal transduction pathways which are believed to ultimately cause mitogenesis and to bind to the corresponding receptors by interacting with specific receptor tyrosine phosphorylation sites via its SH2 domains (31, 35). However, in the case of the IFNα/β receptor, PTP1D was found to associate even with the unphosphorylated receptor or with a GST fusion protein containing the cytoplasmic region of the IFNα/β receptor, suggesting that mechanisms other then phosphotyrosine/SH2 domain interaction can account for PTP1D binding to a receptor. Whether the interaction is direct or involves intermediate docking proteins remains to be clarified. The target proteins that become dephosphorylated by PTP1D also remain unclear. With respect to the activation of the Jak/Stat pathway by IFNα/β, we suspect that Tyk2 is a possible target of PTP1D as we and others consistently observed this member of the Jak family of tyrosine kinases to be constitutively tyrosine-phosphorylated (34, 36, 37). It is possible that Tyk2 becomes activated via a similar mechanism as Src-like tyrosine kinases, namely by undergoing dephosphorylation of an inhibitory phosphotyrosine residue. Experiments to identify the site of basal Tyk2 tyrosine phosphorylation and to determine whether this site is indeed the target for PTP1D are currently in progress.

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