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Identification of Shp-2 as a Stat5A Phosphatase*

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Stat5A, a member of the signal transducers and activators of transcription (Stat) family, is activated upon a single tyrosine phosphorylation. Although much is known about the activation process, the mechanism by which the tyrosine-phosphorylated Stat5A proteins are inactivated is largely unknown. In this report, we demonstrate that down-regulation of the tyrosine-phosphorylated Stat5A was via dephosphorylation. Using tyrosine-phosphorylated peptides derived from Stat5A, we were able to purify protein-tyrosine phosphatase Shp-2 from cell lysates. Shp-2, but not Shp-1, specifically interacted with Stat5A *in vivo*, and the interaction was tyrosine phosphorylation-dependent. Moreover, Shp-2 was able to accelerate Stat5A dephosphorylation, and dephosphorylation of Stat5A was dramatically delayed in Shp-2-deficient cells. Therefore, we conclude that Shp-2 is a Stat5A phosphatase, which down-regulates the active Stat5A *in vivo*.

Cytokines regulate a variety of cellular responses, including cell growth, survival, differentiation, and function and exert their diverse effects through interaction with specific receptors (1, 2). Receptor aggregation, as a result of cytokine binding, activates one or more members of the receptor-associated Janus family of protein-tyrosine kinases (Jaks)[1] (1, 2). Activated Jaks phosphorylate specific tyrosine residues on the receptor, which serve as docking sites to recruit a variety of signaling molecules, such as signal transducers and activators of transcription (Stats) (1, 3). Stat proteins exist normally as latent monomers in the cytoplasm and activation of Stat is totally dependent upon a single tyrosine phosphorylation by Jaks. Once phosphorylated, the Stats dimerize, translocate to the nucleus, and activate a variety of cytokine-inducible genes (1, 3).

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[1] The abbreviations used are: Jak, Janus kinase; IL, interleukin; SOCS, suppressors of cytokine signaling; Stat, signal transducers and activators of transcription; MEF, mouse embryonic fibroblasts; SH2, Src homology domain 2; GST, glutathione *S*-transferase; PTP, protein-tyrosine phosphatase domain; OSM, Oncostatin M.

To date, seven mammalian Stat family members have been identified, including Stat1, 2, 3, 4, 5A, 5B, and 6. Gene disruptions in mice have highlighted unique functions for each Stat family member in cytokine signaling (4). The two closely related Stat5 gene products, Stat5A and Stat5B, have been of particular interest because of the broad spectrum of cytokines that induce their activation by tyrosine phosphorylation. Stat5A and 5B are activated by cytokines that affect the myeloid lineages, including erythropoietin (Epo), thrombopoietin (Tpo), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (5–7), and by cytokines that affect lymphoid lineages, including IL-2, IL-7, IL-9, IL-13, and IL-15 (8–10). In addition, both Stat5 proteins are activated by growth hormone (GH) and prolactin (11, 12). Gene targeting studies have demonstrated that Stat5A plays a critical role in prolactin signaling in the lactating mammary gland, where it is highly expressed relative to Stat5B (13, 14). In contrast, Stat5B functions in GH signaling in the liver, where this isoform is highly expressed (14, 15). In addition, studies of Stat5A/5B nullizygous mice have illustrated a key role of Stat5A and Stat5B in prolactin regulation of ovarian function (14) and IL-2-induced T cell proliferation (16).

The effect of cytokines is modulated in both magnitude and duration; therefore, a braking mechanism must be operative in cytokine signaling. Indeed, several negatively regulatory mechanisms have been identified, which include receptor endocytosis and lysosomal degradation (17), dephosphorylation of receptors and Jak kinases by tyrosine phosphatases (Shp-1 or CD45) (18, 19), and binding of suppressors of cytokine signaling (SOCS) to receptors and Jaks (20). These events explain control at the levels of receptor and Jak kinases. However, little is known about down-regulation of tyrosine-phosphorylated and active Stat proteins. Although a family of protein inhibitors of activated Stats (PIAS) has been shown to inhibit Stat function in the nucleus (21), it is unclear whether a phosphatase or a protease is involved in turnover of Stats. An ubiquitin-dependent proteasome pathway was proposed to mediate Stat1 turnover (22). However, the apparent stabilization of tyrosine-phosphorylated Stat1 by proteasome inhibitors was due to sustained signaling rather than a direct effect on Stat1 turnover (23). Therefore, it is more likely that the turnover of tyrosine-phosphorylated Stat1 is mediated by a phosphatase. In fact, recent studies using cells derived from TC-PTP-deficient mice have clearly demonstrated that TC-PTP is a Stat1 phosphatase (50). In previous experiments, we demonstrated that different mechanisms are involved in regulating the inactivation of various Stat proteins (24). The proteasome inhibitors MG132 and lactacystin inhibited the turnover of tyrosine-phosphorylated forms of Stat4, Stat5, and Stat6, without any

effects on the turnover of tyrosine-phosphorylated Stat1, Stat2, and Stat3. Despite the fact that these two proteasome inhibitors dramatically stabilize the tyrosine-phosphorylated Stat5, there is no direct evidence to support a notion that protein degradation is responsible for the down-regulation of active Stat5 (24). Previous studies with overexpression systems have suggested that several phosphatases, including Shp-2 (25), PTP1B (26), TC-PTP (27), and phosphatase 2A (28), are able to interact and dephosphorylate Stat5A; however, the physiological role of these phosphatases in down-regulation of Stat5A is not clear. In this report, we address the key unanswered question regarding Stat5A down-regulation and identify Shp-2 as a Stat5A phosphatase.

EXPERIMENTAL PROCEDURES

Antibodies, cDNAs, and Inhibitors—Anti-Statl (C24), anti-SHP-1 (C-19), and anti-SHP-2 (C-18) antibodies were purchased from Santa Cruz Biotechnology. Anti-GST (06–332) and anti-phosphotyrosine antibodies (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphoStat5 monoclonal antibodies were purchased from ZYMED Laboratories, Inc. (San Francisco, CA). Antisera against Stat3, Stat5A, and Stat5B, and Jak2 have been described previously (29, 30). The cDNAs encoding murine Jak2, Stat5A, Stat5AY-F_{694}, EpoR, and Shp-2 were subcloned into mammalian expression vectors pRK5 or pcDNA3 (Invitrogen). Protein-tyrosine kinase inhibitor staurosporine was purchased from Sigma.

Cells and Transfection—32D(EpoR wt) and 32D(EpoR H) cells were cultured in RPMI 1640 containing 10% fetal bovine serum and supplemented with murine IL-3 (25 units/ml). COS-7 cells and mouse embryonic fibroblasts (MEF) derived from wild-type or Shp-2 mutant mice were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transfection, COS-7 cells were seeded in 100 or 60-mm tissue culture dishes 24 h before transfection, and subconfluent cells were transfected with various combinations of cDNAs in mammalian expression vectors by LipoFectamine (Invitrogen) according to the manufacturer's instructions. For co-immunoprecipitation experiments, cells were lysed in lysis buffer without phosphatase inhibitors and phosphate salts (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, aprotinin 3 μg/ml, pepstain 2 μg/ml, leupeptin 1 μg/ml) or phosphate buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1 mM Na_3VO_4, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1% Triton X-100, aprotinin 3 μg/ml, pepstain 2 μg/ml, leupeptin 1 μg/ml) 48 h after transfection. For dephosphorylation experiments, MEF cells were stimulated with cytokines and subsequently lysed with SDS-PAGE sample buffer at different time points following cytokine removal.

Pulse Chase Experiments—Log-phase 32D(EpoR H) cells were starved in RPMI 1640 containing 1% fetal bovine serum overnight. Cells were washed twice with phosphate-buffered saline and cultured in methionine-free Dulbecco's modified Eagle's medium at 2×10^7/ml containing 10% dialyzed fetal bovine serum with 100 μCi/ml of [^{35}S]methionine for 3 h. Then, murine IL-3 (25 units/ml) and Epo (20 units/ml) were added for 15 min. Cells were washed three times with phosphate-buffered saline and resuspended at 1×10^6/ml in fresh medium without cytokines. At the times indicated, whole-cell lysate was generated, and immunoprecipitation and SDS-PAGE was carried out. Then the proteins were transferred to nitrocellulose membrane, and the membrane was exposed to film.

Peptide Binding and Mass Spectrometry Analysis—The tyrosine-phosphorylated peptides (TPVLAKAVDG(pY)VKPQIKQ) and the control non-tyrosyl-phosphorylated (TPVLAKAVDGYVKPQIKQ) derived from Stat5A were synthesized and conjugated to Sepharose 4B beads through the primary amino groups at the N terminus of the peptides. 32D (EpoR wt) cells (3×10^8) were lysed in lysis buffer without phosphatase inhibitors and phosphate salts (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, aprotinin 3 μg/ml, pepstain 2 μg/ml, leupeptin 1 μg/ml) at 4 °C. Cell extracts were precleared with Sepharose beads and subsequently incubated with 300 μl of the tyrosine-phosphorylated peptides-conjugated or the non-tyrosine-phosphorylated control peptides-conjugated Sepharose beads at 4 °C for 1 h. After washing three times with cold lysis buffer, the proteins binding to the peptide-conjugated Sepharose beads were eluted in SDS sample buffer at 100 °C for 5 min and were subjected to SDS-PAGE and silver staining. The protein specifically bound to the tyrosine-phosphorylated peptides underwent an *in-gel* tryptic digestion. The peptides derived from the protein band were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis (31). Afterward, the mass spectrometry data were subjected to a search of the NCBInr protein data base with ProteinProspector programs at prospector.ucsf.edu.

PTPase Assays—After binding to cell lysate from 32D (EpoR wt) cells (1×10^7) and a subsequent five-time washing with cold lysis buffer, peptide-conjugated Sepharose beads were incubated in 50 μl of PTPase buffer (50 mM Tris, pH 7.4, 0.2 mM phosphotyrosine peptide substrate) at 22 °C for 1 h of 100 ml of malachite green solution (Upstate Biotechnology, Inc.) was added to each reaction, which was then incubated at 22 °C for 5 min prior to measurement of $OD_{660\ nm}$ to quantify the level of free phosphate cleaved by the PTPases from the substrate.

In Vitro Binding of GST Fusion Proteins—GST fusion proteins GST-SH2, containing both SH2 domains of Shp-2 and GST-PTP, containing the PTP catalytic domain of Shp-2, in pGEX vectors were expressed in *Escherichia coli* and lysed in lysis buffer without phosphatase inhibitors and phosphate salts (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, aprotinin (3 μg/ml), pepstain (2 μg/ml), leupeptin (1 μg/ml)) by sonication and purified by glutathione-Sepharose beads. After washing with cold lysis buffer, the glutathione-Sepharose beads containing comparable GST fusion proteins were incubated with cell lysate from COS-7 cells co-transfected with Stat5A and Jak2. After washing three times, the bound proteins were eluted in SDS sample buffer at 100 °C for 5 min and were subjected to SDS-PAGE and Western blot analysis with anti-GST or anti-Stat5A antibodies.

RESULTS

Down-regulation of the Tyrosine-phosphorylated Stat5A Is via Dephosphorylation—Stat5A is activated by IL-3 in IL-3-dependent myeloid cells 32D (EpoR wt), which also express wild-type erythropoietin receptors (EpoR wt), and removal of IL-3 leads to the disappearance of the tyrosine-phosphorylated Stat5 (24, 29). However, only a small fraction of Stat5 is activated by tyrosine phosphorylation following treatment of 32D (EpoR wt) cells with IL-3, which makes it difficult to investigate down-regulation of the tyrosine-phosphorylated Stat5 (24). To overcome this problem, we employed a variant of IL-3-dependent 32D myeloid cell line, 32D (EpoR H), which expresses a truncated form of the EpoR. Due to elimination of the carboxyl-terminal region of the EpoR cytoplasmic domain, which is required for negative regulation of Epo signaling by recruiting Shp-1, Jak2 activation is more sustained in 32D (EpoR H), relative to 32D (EpoR wt), cells upon exposure to Epo (32). Stimulation of these cells, 32D (EpoR H) with both IL-3 and Epo, is expected to induce tyrosine phosphorylation of relatively larger amounts of Stat5.

32D (EpoR H) cells were starved, pulse-labeled with [^{35}S]methionine, and then stimulated with both IL-3 and Epo. After removal of the cytokines, levels of phosphorylated Stat5A were examined at different time points. IL-3 plus Epo induced tyrosine phosphorylation of substantial amounts of Stat5A protein as indicated by immunoblotting with the anti-pTyr antibodies (Fig. 1*A*). In addition, IL-3 plus Epo induced tyrosine phosphorylation of substantial amounts of Stat5B protein in 32D (EpoR H) cells (data not shown). Interestingly, 4 h after cytokine removal, tyrosine-phosphorylated Stat5A disappeared, as indicated by the absence of the phosphorylated band (Fig. 1*A*). However, the total amount of Stat5A, which is the sum of the phosphorylated and non-phosphorylated forms, remained constant throughout the duration of the experiment as indicated by the invariable densities of [^{35}S]methionine-labeled Stat5A bands in the autoradiography (Fig. 1*A*). Quantitation of the band densities further confirmed that levels of Stat5A proteins remained unchanged throughout the duration of the experiment (Fig. 1*B*). These data provide direct evidence that the down-regulation of tyrosine-phosphorylated Stat5A is mediated by a protein-tyrosine phosphatase.

Shp-2 Specifically Associates with the Tyrosine-phosphorylated Peptide Derived from Stat5A—Phosphorylation of a single tyrosine residue (Tyr-694) of Stat5A results in its activation

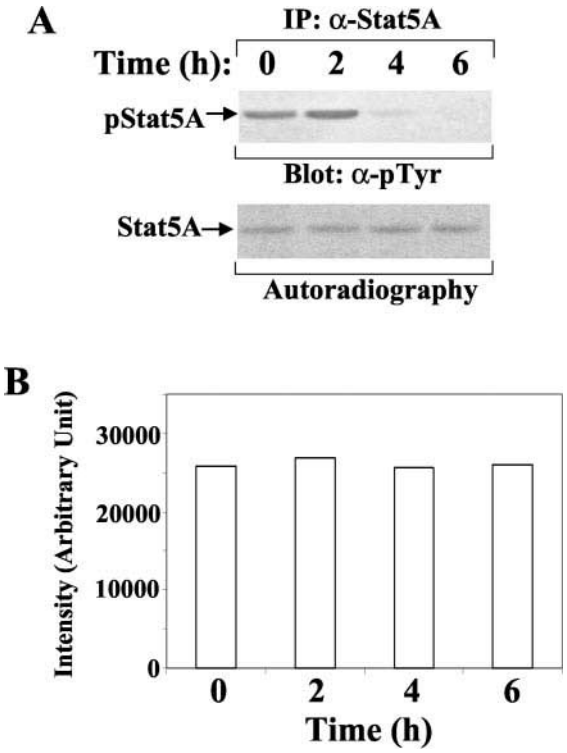

FIG. 1. **Down-regulation of tyrosine-phosphorylated Stat5A is via dephosphorylation.** *A*, analysis of down-regulation of tyrosine-phosphorylated Stat5A with [^{35}S]methionine pulse-labeling. 32D (EpoR H) cells were starved overnight, pulse-labeled with [^{35}S]methionine and stimulated with both IL3 and Epo. After removal of the [^{35}S]methionine and the cytokines, the cells were collected and lysed at the indicated time points. Cell lysates were immunoprecipitated with anti-Stat5A antibodies and precipitated proteins were subjected to SDS-PAGE and subsequently were transferred to a nitrocellulose membrane. The membrane was blotted with anti-phosphotyrosine (α-pTyr) antibody (*upper*) or visualized by autoradiography (*lower*). *B*, quantitation of the band density of total Stat5A. The band densities of total Stat5A shown in *A*, (*lower*) was quantified by densitometry. The figure shown is representative of three independent experiments.

(29); therefore, the phosphatase responsible for Stat5A dephosphorylation must recognize Tyr-694 of Stat5A. The amino acid sequence surrounding a target phosphotyrosine plays a critical role in determining the specificity and avidity of the interaction between a phosphatase and its substrate (33). Therefore, we utilized tyrosine-phosphorylated peptides corresponding to the sequence surrounding Tyr-694 of Stat5A to identify the phosphatase capable of interacting with tyrosine-phosphorylated Stat5A. Tyrosine-phosphorylated peptides and their corresponding non-phosphorylated control peptides were conjugated to Sepharose beads. As the phosphorylated Tyr-694 (pTyr-694) on one Stat5A molecule is able to interact with the SH2 domain on another Stat5A molecule, resulting in a homodimerization, we first examined the ability of the phosphorylated and non-phosphorylated Stat5A peptides to specifically associate with Stat5A itself, the known Stat5A physiological partner. Cell extracts from 32D (EpoR wt) myeloid cells were incubated with peptide-conjugated Sepharose beads. After extensive washing, bound proteins were eluted from the beads and subjected to SDS-PAGE and Western blot analysis for Stat5A. Stat5A associated with the tyrosine-phosphorylated Stat5A peptides but not the non-phosphorylated control peptides (Fig. 2*A*). In contrast, Stat1 molecules, which do not dimerize with Stat5A, did not bind to the tyrosine-phosphorylated peptides even though they were present at readily detectable levels (Fig. 2*A*).

Next, we examined whether tyrosine-phosphorylated Stat5A peptide-conjugated Sepharose beads could recruit a protein-tyrosine phosphatase. Cell extracts from 32D (EpoR wt) myeloid cells were preincubated with unconjugated Sepharose beads to remove nonspecific binding proteins. Subsequently, precleared supernatants were incubated with Sepharose beads conjugated with tyrosine-phosphorylated or non-phosphorylated Stat5A peptides. After extensive washing, the beads were subjected to an *in vitro* protein phosphatase assay. Significantly more protein phosphatase activity was associated with beads conjugated with tyrosine-phosphorylated peptides relative to those conjugated with non-phosphorylated control peptides (Fig. 2*B*). Therefore, peptides containing the phosphorylated tyrosine residue within Stat5A bind a protein-tyrosine phosphatase.

To identify the potential Stat5A phosphatase, we initiated a large scale screening for proteins that specifically bind to Stat5A phosphopeptides. To permit the potential phosphatase to bind to the tyrosine-phosphorylated peptides, 32D (EpoR wt) myeloid cells were lysed in lysis buffer without phosphatase inhibitors and phosphate salts. All procedures were carried out at 4 °C to avoid dephosphorylation of the phosphorylated peptides. Cell extracts were precleared with unconjugated Sepharose beads and subsequently incubated with Sepharose beads conjugated with tyrosine-phosphorylated peptides or with non-phosphorylated control peptides. After extensive washing, proteins bound to the beads were eluted and subjected to SDS-PAGE and silver staining. A protein migrating at an apparent molecular mass of ~70 kDa specifically bound to the tyrosine-phosphorylated, but not control, peptides (Fig. 2*C*). Interestingly, binding of the 70 kDa protein could only be detected in the Tris-HCl buffer, which had neither phosphate salts nor phosphatase inhibitors (Fig. 2*C*). This suggests that the interaction between the 70 kDa protein and the tyrosine-phosphorylated peptides was not likely due to an interaction between an SH2 domain and a phosphotyrosine *in vitro*, although weak interaction of an SH2 domain with a phosphotyrosine may be blocked by excess amount of phosphate or its analogs. Following an in-gel tryptic digestion, the peptides derived from the 70 kDa protein were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis (31). The resulting mass spectrometry data (not shown) were used to search the NCBInr protein data bases with ProteinProspector software (prospector.ucsf.edu), identifying the 70-kDa protein as a known SH2 domain-containing protein-tyrosine phosphatase, Shp-2. The identity of this protein was further confirmed by immunoblot analysis using a specific anti-Shp-2 antibody (Fig. 2*D*). Notably, the other mammalian SH2 domain-containing protein-tyrosine phosphatase, Shp-1, bound to neither the tyrosine-phosphorylated peptides nor the non-phosphorylated control peptides even though both Shp-1 and Shp-2 proteins were highly expressed in the 32D (EpoR wt) cells (Fig. 2*D*). Therefore, Shp-2 specifically associates with the tyrosine-phosphorylated peptides corresponding to the tyrosine-phosphorylation site within Stat5A.

Shp-2 Associates with the Tyrosine-phosphorylated Stat5A in Vivo—The next critical issue we addressed was whether Shp-2 interacts with tyrosine-phosphorylated Stat5A in cells. Shp-2 and Stat5A were co-expressed in COS-7 cells in the presence or absence of Jak2 kinase. As expected, overexpression of Jak2 induced tyrosine phosphorylation of Stat5A (Fig. 3*A*, *upper panel*), and the tyrosine phosphorylation was abolished when the critical Tyr-694 of Stat5A was mutated to phenylalanine (Fig. 3*A*, *upper panel*). To determine whether Stat5A and Shp-2 form a complex, Shp-2 immunoprecipitates from lysates of COS-7 transfectants were subjected to SDS-PAGE and Western blot analysis with anti-Stat5A antibodies. Stat5A stably associated with Shp-2 in the presence of Jak2 and, to a much

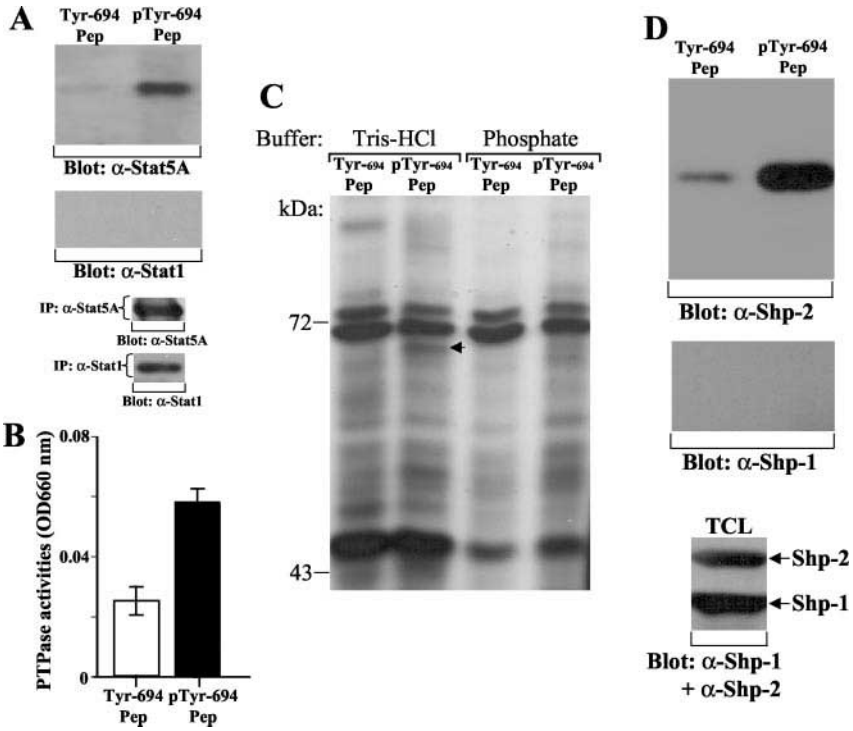

FIG. 2. **Shp-2 associates with the phosphopeptides corresponding to the tyrosine phosphorylation site within Stat5A.** *A*, association of Stat5A with the tyrosine-phosphorylated Stat5A peptides. 32D (EpoR wt) cells (1×10^7) were lysed in lysis buffer without phosphatase inhibitors and phosphate salts at 4 °C. Cell lysates were incubated with Sepharose beads conjugated with the tyrosine-phosphorylated peptides (pTyr-694 Pep) or the non-phosphorylated control peptides (Tyr-694 Pep). Bound proteins were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-Stat5A or anti-Stat1 antibodies (*upper*). The presence of Stat5A and Stat1 in 32D (EpoR wt) cell lysates was confirmed by immunoprecipitation and Western blot analysis with anti-Stat5A or anti-Stat1 antibodies, respectively (*lower*). *B*, association of protein-tyrosine phosphatase activity with the tyrosine-phosphorylated Stat5A peptides. 32D (EpoR wt) cells (1×10^7) were lysed in lysis buffer without phosphatase inhibitors and phosphate salts at 4 °C. Cell lysates were incubated with Sepharose beads conjugated with the tyrosine-phosphorylated peptides (pTyr-694 Pep) or the non-phosphorylated control peptides (Tyr-694 Pep). After extensive washing, the peptide-conjugated Sepharose beads were subjected to an *in vitro* protein-tyrosine phosphatase assay. *C*, association of a 70-kDa protein with the tyrosine-phosphorylated Stat5A peptides. 32D (EpoR wt) cells (5×10^8) were lysed in lysis buffer with (phosphate) or without phosphatase inhibitors and phosphate salts (Tris-HCl) at 4 °C. Cell lysates were precleared with unconjugated Sepharose beads and subsequently incubated with Sepharose beads conjugated with the tyrosine-phosphorylated peptides (pTyr-694 Pep) or the non-phosphorylated control peptides (Tyr-694 Pep) at 4 °C for 1 h. After washing, bound proteins were eluted and subjected to SDS-PAGE followed by silver staining. A protein that migrated at apparent molecular weight of 70 kDa (*arrow*) specifically bound to the tyrosine-phosphorylated Stat5A peptides only in Tris-HCl lysis buffer. *D*, confirmation of the 70-kDa protein as Shp-2 by Western blot analysis. 32D (EpoR wt) cells (1×10^7) were lysed in lysis buffer without phosphatase inhibitors and phosphate salts at 4 °C. Cell lysates were incubated with Sepharose beads conjugated with the tyrosine-phosphorylated Stat5A peptides (pTyr-694 Pep) or the non-phosphorylated control peptides (Tyr-694 Pep). Bound proteins were subjected to Western blot analysis with anti-Shp-2 or anti-Shp-1 antibodies (*upper*). The presence of Shp-2 and Shp-1 in 32D (EpoR wt) cell lysates were confirmed by the immunoprecipitation and Western blot analysis with anti-Shp-2 or anti-Shp-1 antibodies, respectively (*lower*).

lesser extent, in the absence of Jak2 (Fig. 3*A*, *lower panel*). Stat5A failed to co-precipitate with Shp-2 when Tyr-694 of Stat5A was mutated to phenylalanine (Fig. 3*A*, *lower panel*). Therefore, Shp-2 associates with Stat5A in a manner that is dependent on phosphorylation of the critical Tyr-694 of Stat5A.

We next examined whether endogenous Shp-2 associates with tyrosine-phosphorylated Stat5A under more physiological conditions. The IL-3-dependent 32D (EpoR wt) cells were cultured in IL-3-containing media or starved in media without IL-3 and subsequently lysed. As expected, tyrosine-phosphorylated Stat5A could be readily detected in the presence of IL-3 but not in its absence (Fig. 3*B*, *upper panel*). Interestingly, co-immunoprecipitation of Shp-2 and Stat5A was readily detectable in lysates of IL-3-treated cells, whereas this interaction was barely detected in starved cells (Fig. 3*B*, *lower panel*). The association of Stat5A with Shp-2 could only be detected in Tris-HCl buffer without phosphate salts and phosphatase inhibitors (Fig. 3*B*, *lower panel*). Additionally, Stat5A did not associate with Shp-1 under the same conditions (Fig. 3*B*, *lower panel*). These results demonstrate that tyrosine-phosphorylated Stat5A specifically associates with endogenous Shp-2 under physiologically relevant conditions.

To localize the structural domains of Shp-2 involved in association with Stat5A, we examined the ability of GST fusion proteins, containing either the two SH2 domains of Shp-2 (GST-SH2) or the protein-tyrosine phosphatase domain (PTP) of Shp-2 (GST-PTP) (Fig. 4*A*), to bind tyrosine-phosphorylated Stat5A. Glutathione-Sepharose beads-loaded with the same amounts of GST-SH2 or GST-PTP fusion proteins were incubated with lysates of COS-7 cells that had been co-transfected with Stat5A and Jak2. The amount of tyrosine-phosphorylated Stat5A bound to the GST fusion proteins was assessed by Western blot analysis. The Shp-2 PTP domain fusion proteins associated with tyrosine-phosphorylated Stat5A whereas the Shp-2 SH2 domain fusion protein failed to (Fig. 4*B*). This same Shp-2 SH2 domain fusion protein has been shown to be able to bind growth hormone receptor (34). Therefore, the PTP catalytic domain, rather than the SH2 domains, of Shp-2 is primarily involved in association with tyrosine-phosphorylated Stat5A.

Shp-2 Dephosphorylates Tyrosine-phosphorylated Stat5A in Vivo—To determine whether Shp-2 dephosphorylates tyrosine-phosphorylated Stat5A in cells, we tested the ability of Shp-2 to attenuate Epo-induced tyrosine phosphorylation of Stat5A. Stat5A and EpoR cDNAs were co-transfected into COS-7 cells with or without Shp-2. 48 h later, cells were stimulated with

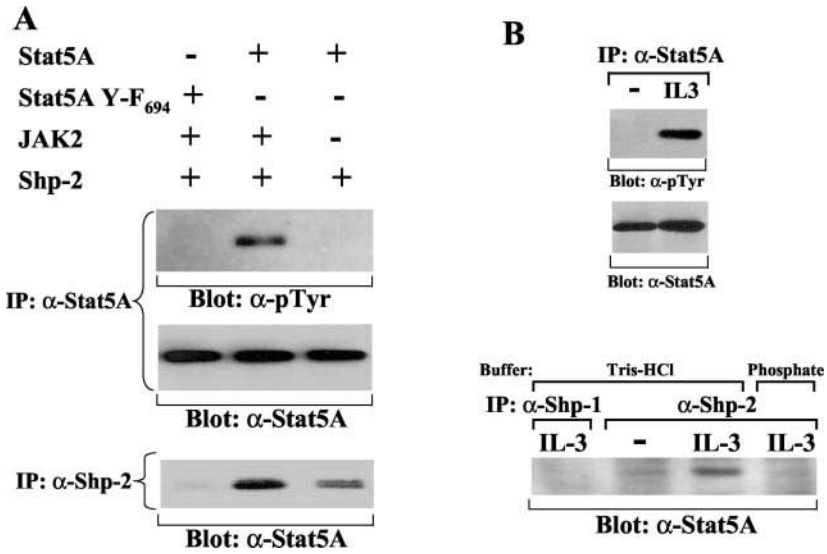

FIG. 3. **Shp-2 associates with the tyrosine-phosphorylated Stat5A *in vivo*.** *A*, association of Shp-2 with Stat5A depending on phosphorylation of Tyr-694. cDNAs of wild-type Stat5A or a mutant form of Stat5A, in which Tyr-694 was substituted with phenylalanine (Y-F694), and cDNAs of Shp-2 were co-transfected into COS-7 cells in the presence or absence of Jak2-encoding cDNAs. After transfection, COS-7 cells were lysed in the lysis buffer without phosphatase inhibitors and phosphate salts. Cell lysates were immunoprecipitated with anti-Shp-2 or anti-Stat5A antibodies. Precipitated proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The proteins immunoprecipitated with anti-Stat5A were blotted with anti-phosphotyrosine (α-*pTyr*) or anti-Stat5A (α-Stat5A) (*upper*) whereas the proteins immunoprecipitated with anti-Shp-2 antibodies were blotted with anti-Stat5A (α-*Stat5A*) (*lower*). *B*, interaction between endogenous Shp-2 and Stat5A depending on phosphorylation of Stat5A. 32D (EpoR wt) cells cultured in medium containing IL-3 (IL-3) or starved (−) overnight were lysed in lysis buffer with (phosphate) or without (Tris-HCl) phosphatase inhibitors and phosphate salts at 4 °C. Cell lysates were immunoprecipitated with anti-Shp-1, anti-Shp-2, or anti-Stat5A. Precipitated proteins were subjected to Western blot analysis. The proteins immunoprecipitated with anti-Stat5A were blotted with anti-phosphotyrosine (α-pTyr) or anti-Stat5A (α-Stat5A) antibodies (*upper*) whereas the proteins immunoprecipitated with anti-Shp-1 or anti-Shp-2 antibodies were blotted with anti-Stat5A (α-Stat5A) antibodies (*lower*). The figure shown is representative of three independent experiments.

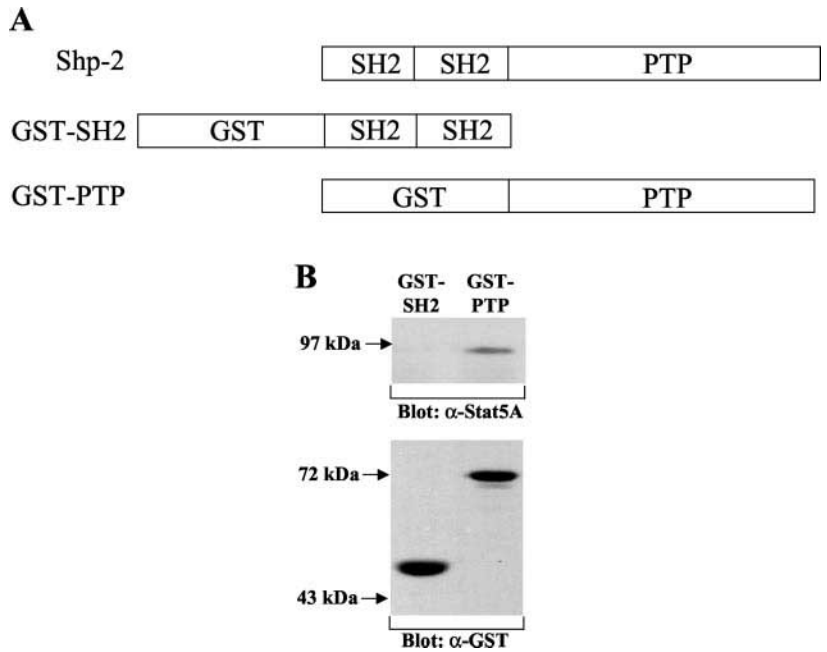

FIG. 4. **The PTP catalytic domain rather than the SH2 domains of Shp-2 is primarily involved in association with tyrosine-phosphorylated Stat5A.** *A*, a schematic diagram of GST fusion proteins containing the two SH2 domains (GST-SH2) or the PTP catalytic domain (GST-PTP) of Shp-2. *B*, association of tyrosine-phosphorylated Stat5A with GST-PTP fusion proteins. GST-SH2 or GST-PTP fusion proteins were incubated with lysates of COS-7 cells that had been co-transfected with Stat5A and Jak2. The amount of tyrosine-phosphorylated Stat5A bound to the GST fusion proteins was assessed by Western blot analysis with anti-Stat5A antibodies (*upper*). The amount of GST fusion proteins was assessed by Western blot analysis with anti-GST antibodies (*lower*). The figure shown is representative of two independent experiments.

Epo for 30 min and subsequently lysed. The level of Epo-induced tyrosine phosphorylation of Stat5A was examined. Overexpression of Shp-2 significantly attenuated Epo-induced tyrosine phosphorylation of Stat5A (Fig. 5*A*). To further determine the role of Shp-2 in dephosphorylation of Stat5A, we examined dephosphorylation of Stat5A in wild-type and Shp-2-deficient (Shp-$2^{-/-}$) fibroblast cells derived from embryos of mice with a targeted deletion of exon 3 of Shp-2 (35). These MEFs were engineered to stably express Epo receptors and Stat5A molecules, and the cells were stimulated with Epo for 15 min. After the cytokine was removed, a tyrosine kinase inhibitor, staurosporine, was added to the cells. Levels of ty-

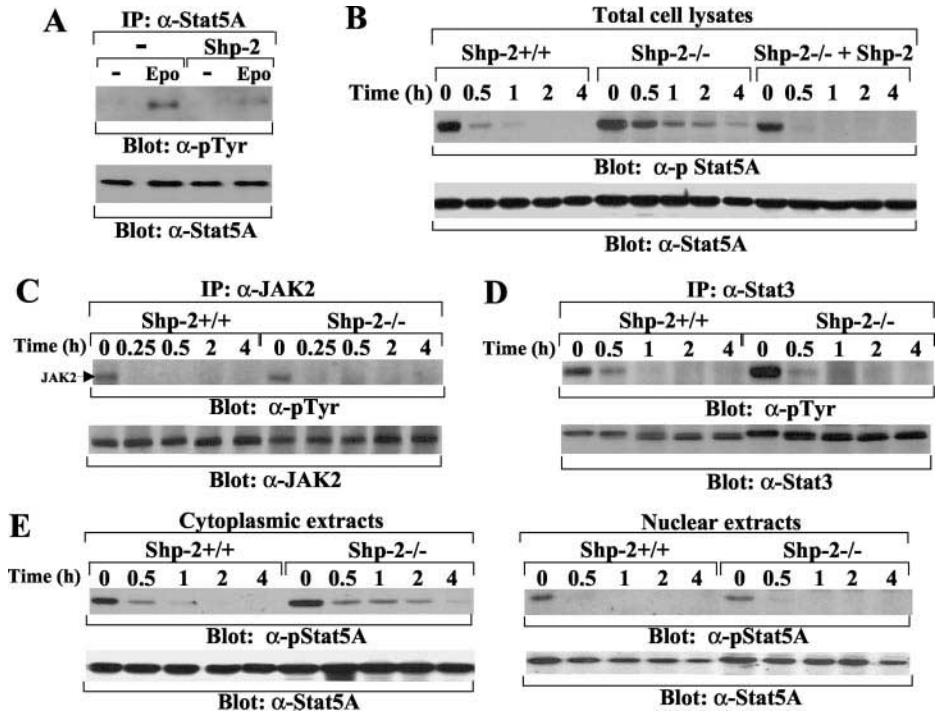

FIG. 5. **Shp-2 is involved in Stat5A dephosphorylation *in vivo*.** *A*, overexpression of Shp-2 attenuating Epo-induced tyrosine phosphorylation of Stat5A. Epo receptor and Stat5A cDNAs were co-transfected into COS-7 cells in the presence or absence of Shp-2. Forty-eight hours after transfection, COS-7 cells were stimulated with Epo for 30 min and subsequently lysed. Cell lysates were immunoprecipitated with anti-Stat5A antibodies. Precipitated proteins were subjected to Western blot analysis with anti-phosphotyrosine (α-*pTyr*) or anti-Stat5A (α-*Stat5A*) antibodies. *B*, dramatically delayed dephosphorylation of Stat5A in Shp-2-deficient fibroblasts. Epo receptor and Stat5A cDNAs were stably expressed in wild-type (Shp-$2^{+/+}$) or Shp-2-deficient (Shp-$2^{-/-}$) MEF or in Shp-2-deficient MEF, in which Shp-2 was re-introduced back via transient transfection (Shp-$2^{-/-}$ + Shp-2). These MEFs were stimulated with Epo for 15 min, and subsequently Epo was removed. At different time points following cytokine removal, cells were collected and lysed. Cell lysates were subjected to Western blot analysis with anti-phosphoStat5 (α-pStat5) or anti-Stat5A (α-Stat5A) antibodies. *C*, Shp-2 deficiency having no effect on the turnover rate of active Jak2 kinase. Wild-type (Shp-$2^{+/+}$) or Shp-2-deficient (Shp-$2^{-/-}$) MEFs were stimulated with Epo as described in *B*. Cell lysates were immunoprecipitated with anti-Jak2 antibodies. Precipitated proteins were subjected to Western blot analysis with anti-phosphotyrosine (α-pTyr) or anti-Jak2 (α-Jak2) antibodies. *D*, comparable rates of dephosphorylation of Stat3 in wild-type and Shp-2 mutant MEFs. Wild-type (Shp-$2^{+/+}$) or Shp-2 mutant (Shp-$2^{-/-}$) MEFs were stimulated with Oncostatin M (OSM) for 15 min, and subsequently OSM was removed. At different time points following cytokine removal, cells were collected and lysed. Cell lysates were immunoprecipitated with anti-Stat3 antibodies. Precipitated proteins were subjected to Western blot analysis with anti-phosphotyrosine (α-pTyr) or anti-Stat3 antibodies. *E*, delayed dephosphorylation of Stat5A in the cytoplasma of Shp-2-deficient MEFs. Wild-type (Shp-$2^{+/+}$) or Shp-2-deficient (Shp-$2^{-/-}$) MEFs were stimulated with Epo as described in *B*. Cytoplasmic and nuclear extracts from these MEFs were subjected to Western blot analysis with anti-phosphoStat5 (α-pStat5) or anti-Stat5A (α-Stat5A) antibodies. The figure shown is representative of three independent experiments.

rosine-phosphorylated Stat5A were assessed at different time points following cytokine removal. Decreased levels of tyrosine-phosphorylated Stat5A were observed within 30 min, and tyrosine-phosphorylated Stat5A was barely detectable within 1 h of cytokine removal in wild-type MEFs (Fig. 5*B*). In contrast, the rate of dephosphorylation of Stat5A was dramatically delayed in Shp-$2^{-/-}$ MEFs, in which tyrosine-phosphorylated Stat5A was detectable even at 4 h following cytokine removal (Fig. 5*B*). To determine that the observed delay in Stat5A dephosphorylation in Shp-$2^{-/-}$ MEFs is due to the lack of Shp-2 protein, we performed reconstitution analysis. Shp-2 was transiently introduced into the Epo receptor and Stat5A expressing Shp-$2^{-/-}$ MEFs via transient transfection. Reintroduction of Shp-2 into Shp-$2^{-/-}$ MEFs was sufficient to restore Stat5A dephosphorylation (Fig. 5*B*). Therefore, Shp-2 is indeed required for the dephosphorylation of Stat5A. To demonstrate that the delayed dephosphorylation of Stat5A in Shp-$2^{-/-}$ MEFs was not due to the delayed down-regulation of Jak2 activity, we examined the turnover rate of active Jak2 kinases. The turnover rate of active Jak2 kinases, indicated by the disappearance of tyrosine-phosphorylated forms, was comparable in both wild-type and Shp-$2^{-/-}$ MEFs (Fig. 5*C*). Thus, the delayed dephosphorylation of Stat5A in Shp-$2^{-/-}$ MEFs was not due to the delayed turnover of Jak2 activity. To determine whether delayed dephosphorylation of Stat5A in Shp-$2^{-/-}$ cells was Stat5A-specific, the rate of dephosphorylation of Stat3 was also assessed. Wild-type and Shp-$2^{-/-}$ MEFs, which express both Stat3 and Oncostatin M (OSM) receptors, were stimulated for 15 min with OSM, a cytokine which potently activates Stat3 (36). The levels of tyrosine-phosphorylated Stat3 were assessed at different time points following cytokine removal. The rate of dephosphorylation of tyrosine-phosphorylated Stat3 was comparable in wild-type and Shp-$2^{-/-}$ MEFs (Fig. 5*D*), which demonstrates that Shp-2 is not involved in the dephosphorylation of Stat3 molecules. To determine the cellular compartment, in which Shp-2 dephosphorylates Stat5A, we examined Stat5A dephosphorylation in cytoplasmic and nuclear fractions derived from wild-type and Shp-2-deficient MEFs treated with Epo followed by removal of the cytokine. Interestingly, the Shp-2-deficiency delayed dephosphorylation of Stat5A mainly in the cytoplasm (Fig. 5*E*). We conclude from these studies that Shp-2 is a Stat5A phosphatase and is specifically involved in dephosphorylation of the tyrosine-phosphorylated Stat5A in cytoplasm.

DISCUSSION

Shp-2 is an SH2 domain-containing tyrosine phosphatase that is widely expressed in all tissues (37, 38), similar to Stat5A (12). Shp-2 appears to be involved in multiple signaling pathways as a positive or a negative regulator (38, 39). Targeted

deletion of Shp-2 in mice resulted in early embryonic lethality (35). Several putative substrates of Shp-2 have been identified, including SHPS-1/SIRPα (40), PZR (41), PDGF-Rβ (42), Gab1 (43), and IRS1 (44). Shp-2 has been shown to interact with Jak kinases (45, 46) and, in Shp-2 mutant cells, tyrosine phosphorylation of Jak1 but not Jak2 was significantly enhanced upon IFN-γ stimulation (47). Nonetheless, phosphorylated Jak kinases appear not to be substrates of Shp-2 (48). Consistently, we show here that Shp-2 deficiency did not influence the turnover of tyrosine-phosphorylated Jak2 upon Epo stimulation (Fig. 5*B*). Jak2 might be the substrate of Shp-1 (18) and the interaction between Jak2 and Shp-1 is direct and independent of SH2 domain-phosphotyrosine interaction (49). We show in the present studies that Shp-2, but not Shp-1, specifically interacts with Stat5A *in vivo* and this interaction is tyrosine phosphorylation-dependent. The functional relevance of this interaction was established in experiments that showed that overexpression of Shp-2 impaired Epo-induced tyrosine phosphorylation of Stat5A and that Shp-2 deficiency dramatically delayed dephosphorylation of Stat5A following cytokine removal. Nonetheless, in Shp-2-deficient cells, Stat5A is still dephosphorylated, albeit delayed. It is possible that there is another phosphatase(s) involved in the dephosphorylation of Stat5A. Previous studies with overexpression systems have suggested that other phosphatases, PTP1B (26), TC-PTP (27), and phosphatase 2A (28), in addition to Shp-2 (25), are able to interact and dephosphorylate Stat5A. However, the physiological role of these phosphatases in down-regulation of Stat5A is not clear. In fact, recent studies using TC-PTP-deficient cells have clearly demonstrated that TC-PTP is a Stat1, but not a Stat5 phosphatase (50). Although our findings provide direct evidence that Shp-2 is a Stat5A phosphatase *in vivo*, the possibility that there is another Stat5A phosphate(s) still exists.

What is the effect of Shp-2 deficiency-caused delay of Stat5A dephosphorylation on the function of Stat5? We examined the induction of *CIS* or *OSM*, two genes that are regulated by Stat5, in Shp-2-deficient MEFs. Interestingly, delayed-dephosphorylation of Stat5A in Shp-2-deficient MEFs did not enhance or extend the induction of the two Stat5 target genes (data not shown). This result is not a total surprise. Continuous treatment of cells with IL-3 or Epo prolongs the phosphorylation of Stat5, but fails to extend the induction of Stat5 target genes (29). In addition, our previous studies (24) have shown that a proteasome inhibitor, MG132, blocks dephosphorylation of both Stat5A and Stat5B, but does not detectably affect the induction of CIS or OSM (data not shown). Therefore, Stat5 must cooperate with additional transcription factor(s), whose activity is only transiently up-regulated by cytokines, to induce Stat5 target genes. Prolonged phosphorylation of Stat5 alone is not sufficient to extend induction of Stat5 target genes.

The molecular basis for the Stat5A and Shp-2 interaction is not fully understood. SH2 domains of Shp-2 have been shown to be important in many cases for subcellular localization of the phosphatase to its substrates (51, 52). However, our findings here demonstrate that the PTP catalytic domain of Shp-2 interacts directly with the sole phosphotyrosine residue of Stat5A while the SH2 domains of Shp-2 appear not to be directly involved in the interaction between the enzyme and this substrate (Fig. 4). An important factor that contributed to our successful identification of Shp-2 as a Stat5A phosphatase might be the right choice of buffer condition used in this study. Phosphate salt, sodium pyrophosphate (30 mM), but not sodium vanadate, is sufficient to block the interaction between Shp-2 and phosphorylated Stat5A (data not shown). However, in the presence of phosphatase inhibitors and phosphate salts, only the catalytically inactive Cys-to-Ser mutant Shp-2, not wild-type, interacts with tyrosine-phosphorylated Stat5 (25). In addition, at a low temperature, ionic and hydrophilic interaction might stabilize the association of the phosphotyrosine of Stat5A with the catalytic domain of Shp-2, which might also contribute to our identification of Shp-2.

In previous studies, we identified a relatively small and potential amphipathic helical region of the carboxyl domain of Stat5A that is important in the control of Stat5A dephosphorylation (24). Therefore, the small region of the carboxyl domain and the phosphotyrosine of Stat5A participate in contact with Shp-2. Interestingly, this region is also a transcriptional activation domain (24). Nevertheless, fractionation of wild-type and Shp-2-deficient MEFs treated with Epo followed by a removal of the cytokine reveals a delay of Stat5A dephosphorylation mainly in the cytoplasmic fraction of Shp-2-deficient MEFs (Fig. 5*E*). Moreover, we have demonstrated that DNA binding is not required for the down-regulation of tyrosine-phosphorylated Stat5A, and that the tyrosine-phosphorylated amino-terminal deletion mutant of Stat5A, which is defective in nuclear translocation, is recycled normally as wild-type Stat5A (24) (data not shown). Our findings agree with other studies, which have also shown that down-regulation of phosphorylated Stat5A can occur in the cytoplasm (25). Therefore, it is more likely that the small region of the carboxyl domain of Stat5A independently recruits a transcriptional complex and Shp-2, although the possibility that Shp-2 exists in the transcriptional activation complex could not be excluded. Given the fact that Shp-2 is mainly distributed in the cytoplasm (53), it is reasonable that dephosphorylation of Stat5A primarily occurs in cytoplasm. However, the possibility that a yet unidentified nuclear phosphatase dephosphorylates Stat5A in the nucleus still exists. In this regard, a recently identified Stat1 phosphatase, TC-PTP, dephosphorylates Stat1 in both nucleus and cytoplasm (50).

Our previous experiments have indicated that different mechanisms are involved in the turnover of different tyrosine-phosphorylated Stat molecules. The difference is conferred by the carboxyl domains of Stat proteins because the Stat5A phenotype of stability in the presence of proteasome inhibitor (MG132) can be transferred onto Statl by simply replacing its carboxyl terminus with that of Stat5A (24). Our current findings that Shp-2 deficiency delays down-regulation of Stat5A but not Stat3 (Fig. 5*C*) further support the notion that the dephosphorylation of various tyrosine-phosphorylated Stats is executed by different phosphatases. The carboxyl domains of different Stats might determine which phosphatase is recruited for their dephosphorylation. It will be interesting to examine the effect of Shp-2 deficiency on the down-regulation of other Stats, especially Stat4 and Stat6.

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