Interactions between SHP-2 phosphotyrosine phosphatase and JAK tyrosine kinases have recently been implicated in cytokine signal transduction. However, the molecular basis of these interactions is not well understood. In this study, we demonstrate that SHP-2 is tyrosine-phosphorylated by and associated with JAK1 and JAK2 but not JAK3 in COS-1 cell cotransfection experiments. SHP-2 phosphatase activity appears not to be required for JAK and SHP-2 interactions because SHP-2 with a mutation at amino acid 463 from Cys to Ser, which renders SHP-2 inactive, can still bind JAKs. We further demonstrate that SHP-2 SH2 domains (amino acids 1–209) are not essential for the association of JAKs with SHP-2, and the region between amino acids 232 and 272 in SHP-2 is important for the interactions. Furthermore, tyrosine residues 304 and 327 in SHP-2 are phosphorylated by JAKs, and phosphorylated SHP-2 can associate with the downstream adapter protein Grb2. Finally, deletion of the N terminus but not the kinase-like domain of JAK2 abolishes the association of JAK2 with SHP-2. Taken together, these studies identified novel sequences for SHP-2 and JAK interactions that suggest unique signaling mechanisms mediated by these two molecules.

SHP-2 (previously known as Syp, SHPTP-2, SHPTP-3, PTP2C, and PTP1D) is a cytoplasmic protein-tyrosine phosphatase containing two Src homology 2 (SH2)1 domains (1–5). Studies have shown that SHP-2 becomes tyrosine-phosphorylated following ligation of various tyrosine kinase receptors (1, 2, 6–12). Recently, tyrosine phosphorylation of SHP-2 has also been observed in signal transduction mediated by non-tyrosine kinase receptors (13–16). In particular, positive regulatory effects of SHP-2 in signaling pathways have been demonstrated in several cytokine systems (10, 16).

Signaling through cytokine receptors is mediated in part by the activation of protein tyrosine kinases, particularly the Janus family kinases (JAKs) (17). The basic characteristics of JAK tyrosine kinases include a C-terminal kinase domain, an adjacent kinase-like domain of unknown function, and an N-terminal JAK homology domain (18). The pivotal roles of JAK kinases in signaling pathways were first shown in the interferon system (19, 20). Subsequent demonstration for the involvement of JAK2 in erythropoietin, growth hormone, and interleukin 3 signaling has established critical roles of JAK kinases in cytokine signal transduction (21–23).

The interactions between protein-tyrosine phosphatase SHP-1 (previously known as PTP1C, SHPTP-1, SHP, and HCP) and JAK2 in erythropoietin signaling have been shown previously (24). The SHP-1 and JAK2 interactions resulted in dephosphorylation of JAK2 and termination of JAK2-mediated signaling (24). Thus, SHP-1 may serve as a negative regulator in erythropoietin signaling. Recently, SHP-2 protein-tyrosine phosphatase has been shown to play positive roles in signal transduction, and the interactions between SHP-2 and JAK kinases have been implicated in signaling pathways mediated by erythropoietin, interleukin 3, interleukin 11, and prolactin (13–16). However, little is known about the molecular basis of interactions between SHP-2 and JAKs. Here we demonstrate the association between JAKs and SHP-2 in COS-1 cell cotransfection experiments and identify novel regions in SHP-2 and JAKs that mediate these interactions.

**EXPERIMENTAL PROCEDURES**

Reagents—Antibodies for JAK1, JAK2, JAK3, and phosphotyrosine were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity-purified anti-Grb2 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies for SHP-2 were produced as described (1). Monoclonal anti-SHP-2 and anti-Flag antibodies were purchased from Transduction Laboratory (Lexington, KY) and Eastman Kodak Company, respectively. ECL detection kit was from Amer sham Corp. N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methy sulfate transfection reagents were purchased from Boehringer Mannheim.

Plasmids and Mutagenesis—The cDNAs encoding murine JAK1, JAK2, JAK3, and SHP-2 were subcloned into mammalian expression vectors pRc/CMV or pCDNA3 (Invitrogen). The C-terminal truncations of SHP-2 were created by polymerase chain reaction and subcloning into pRc/CMV or pCDNA3. Deletion of SH2 domains of SHP-2 was done by subcloning the PstI fragment (amino acids 209–385) into Flag-tagged expression vector pFlag-CMV-2 (Kodak). Phosphatase-inactive mutant of SHP-2 was generated by site-directed mutagenesis (changing amino acid 463 from Cys to Ser). Deletion of the kinase-like domain in JAK2 was obtained by ligation after removing the BglII fragment, which includes the kinase-like domain. The N-terminal deletion of JAK2 was done by restriction enzyme digestion (XhoI/XbaI or KpnI/XhoI), and the fragments from XhoI/XbaI or KpnI/XhoI digestion were subcloned into pFlag-CMV-2. All plasmid constructs were verified by DNA sequencing.

Cell Cultures and Transfections—COS-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum. For cotransfection experiments, COS-1 cells were seeded in 100-mm tissue culture dishes 24 h before transfection, and subconfluent cells were transfected with various combinations of plasmid constructs (see “Results and Discussion”) by the N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methy sulfate method as described previously (25). Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, and 1 mM sodium deoxycholate).

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SHP-2 is tyrosine-phosphorylated by and associated with JAK kinases in COS-1 cells. A and B, tyrosine phosphorylation and association of SHP-2 with JAKs. Wild-type (SHP-2) and phosphatase inactive mutant (SHP-2*) SHP-2 cDNAs (8 and 28). In short, cell lysates from a 100-mm dish were immunoprecipitated with either SHP-2 (1 μl), JAK (1 μl), or Flag (4 μg) antibodies as indicated under “Results and Discussion.” The immunoprecipitates were separated on 8% SDS-polyacrylamide gels, transferred onto PVDF membranes, and immunoblotted with either phosphotyrosine (1 μg/ml), SHP-2 (monoclonal antibodies, 1 μg/ml), JAK (1:2000), or Flag (monoclonal antibodies, 5 μg/ml) antibodies as described under “Results and Discussion.”

**RESULTS AND DISCUSSION**

**SHP-2 Is Tyrosine-phosphorylated by and Associated with JAK1 and JAK2 Kinases—**Recent studies have shown that SHP-2 is tyrosine-phosphorylated in signaling pathways mediated by cytokines that activate JAK family tyrosine kinases (13–16), indicating potential interactions between SHP-2 and JAKs. In the present study, we examined whether SHP-2 can be tyrosine-phosphorylated by JAK non-receptor tyrosine kinases and determined regions required for JAK and SHP-2 interactions. Wild-type SHP-2 cDNA (8 μg/transfection) was cotransfected into COS-1 cells with 15 μg of either JAK1, JAK2, or JAK3 cDNA, and the interactions between SHP-2 and JAKs were analyzed 48 h after transfection. As shown in Fig. 1A, two tyrosine-phosphorylated proteins with molecular masses of 70 and 130 kDa were observed in anti-SHP-2 immunoprecipitation from cells cotransfected with SHP-2 and JAK1 or JAK2 but not JAK3 cDNAs. We further confirmed by immunoblotting that the 70- and 130-kDa tyrosine-phosphorylated proteins are SHP-2 and JAK1 or JAK2, respectively, as shown in the lower panels of Fig. 1A. The inability of SHP-2 to interact with JAK3 was not due to the lack of expression of JAK3 because equal levels of JAK expression were detected in cotransfected COS-1 cells (data not shown). To test whether phosphatase activity of SHP-2 is required for its interactions with JAK kinases, a phosphatase inactive mutant of SHP-2, SHP-2*, was similarly cotransfected with different JAKs into COS-1 cells (Fig. 1B). Fig. 1B shows that the phosphatase activity of SHP-2 is not required for its association with JAK1 and JAK2 because point mutation at amino acid 463 from Cys to Ser, which inactivates SHP-2, did not affect tyrosine phosphorylation and association of SHP-2* with JAK1 or JAK2. In the reciprocal immunoprecipitation as shown in Fig. 1C, anti-JAK1 or anti-JAK2 antibodies immunoprecipitated the 130-kDa tyrosine-phosphorylated JAK1 or JAK2, protein and coprecipitated the 70-kDa tyrosine-phosphorylated SHP-2, although many more cell lysates and antibodies were required. Taken together, these results indicate that SHP-2 and JAKs can specifically associate and form stable complexes in vivo.

It is well known that cytokine receptors and signal transducers and activators of transcription (Stats) are substrates for JAK tyrosine kinases (19). Recent studies have also shown that insulin receptor substrate-1 and -2 are physiological substrates for JAK kinases in certain cytokine signaling (25). Here we demonstrate that SHP-2 is not only a specific substrate for JAK1 and JAK2 in vivo but also is tightly associated with JAK1 and JAK2. The physiological interactions between SHP-2 and JAK kinases implicate important roles in cytokine signaling. For example, SHP-2 and JAK2 interactions have been shown to

2 mM sodium vanadate, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) 48 h after transfection.

**Immunoprecipitation and Immunoblotting—**The procedures for immunoprecipitation and immunoblotting were described previously (26–28). In short, cell lysates from a 100-mm dish were immunoprecipitated with either SHP-2 (1 μl), JAK (1 μl), or Flag (4 μg) antibodies as indicated under “Results and Discussion.” The immunoprecipitates were separated on 8% SDS-polyacrylamide gels, transferred onto PVDF membranes, and immunoblotted with either phosphotyrosine (1 μg/ml), SHP-2 (monoclonal antibodies, 1 μg/ml), JAK (1:2000), or Flag (monoclonal antibodies, 5 μg/ml) antibodies as described under “Results and Discussion.”
be essential for gene expression mediated by prolactin (16). Interestingly, although SHP-2 is tightly associated with JAK1 or JAK2, it appears that phosphorylated JAK kinases may not be good substrates for SHP-2 because the overall level of JAK tyrosine phosphorylation and kinase activity are not significantly different when compared to cotransfection of either wild-type SHP-2 (Fig. 1A) or the phosphatase-inactive SHP-2 mutant (Fig. 1B). JAK2 has also been shown to interact with protein-tyrosine phosphatase SHP-1 in erythropoietin signaling. However, these interactions between JAK2 and SHP-1 result in the dephosphorylation and inactivation of JAK2, indicating that JAK2 is likely to be a potential substrate for SHP-1 (24). These results suggest that SHP-1 may act as a negative regulator, whereas SHP-2 may play positive roles in signal transmission.

Requirement of Amino Acids 232–272 Region but Not SH2 Domains of SHP-2 for the Interactions between SHP-2 and JAKs—To understand the molecular basis of the JAK and SHP-2 interactions, we have constructed several SHP-2 C-terminal truncation mutants (SHP-2m1 to SHP-2m6) as well as a SHP-2 N-terminal deletion mutant (Flag-SHP-2) as indicated in Fig. 2A. Cotransfection of COS-1 cells with wild-type JAKs and the different C-terminal truncations of SHP-2 were then performed. Proteins encoded by different C-terminal truncation mutants of SHP-2 were immunoprecipitated with anti-SHP-2 antibodies and assessed for their status of tyrosine phosphorylation as well as association with JAKs. The results in Fig. 2B indicated that the smallest construct, SHP-2m6 (amino acids 1–232), comprised of only the two SH2 domains of SHP-2, was not tyrosine-phosphorylated and did not associate with JAKs. The inability of SHP-2m6 to associate with JAK was not due to the lack of expression of JAKs because equal levels of JAK were detected in transfected COS-1 cells (data not shown). However, a slightly longer construct SHP-2m4 (amino acids 1–272) formed a stable complex with JAKs, as demonstrated by coimmunoprecipitation (Fig. 2B), indicating that the region between amino acids 232 and 272 is important for SHP-2 and JAK interactions. Interestingly, SHP-2m4 (amino acids 1–293) and SHP-2m5 (amino acids 1–272), although not tyrosine-phosphorylated, are efficiently coimmunoprecipitated with JAK1 and JAK2 (Fig. 2B), implicating that tyrosine phosphorylation of SHP-2 is not essential for its associations with JAKs. These results, therefore, suggest that SH2 domains of SHP-2 are insufficient for the interactions between JAKs and SHP-2, and in fact, it is the region between amino acids 232–272 in SHP-2 that is required for JAK and SHP-2 association. The sequences between amino acids 232 and 272 comprise a linker region between the SH2 domains and the phosphatase domain of SHP-2, which are highly conserved between SHP-1 and SHP-2. Site-directed mutagenesis will be essential to identify residues required for such interactions and to evaluate whether these sequences determine the specificity of SHP-1 and SHP-2.

We have shown that SH2 domains of SHP-2 alone are not sufficient for its association with JAKs; however, these results did not rule out the possibility that SH2 domains plus other regions in SHP-2 may be required for its association with JAKs. To test this possibility, we have constructed a SHP-2 mutant lacking the two SH2 domains at the N terminus, as indicated in Fig. 2A (Flag-SHP-2). Cotransfection experiments of SHP-2 (positive control), Flag-SHP-2, and Flag-bacterial alkaline phosphatase (Flag-BAP; negative control) with JAK2 were performed. As indicated in Fig. 3A, total cell lysates from cotransfected cells were resolved on 8% SDS-PAGE, immobilized on PVDF, and immunoblotted with anti-tyrosine or Flag antibodies. The results demonstrated that SHP-2 and Flag-SHP-2, but not unrelated Flag-BAP, were tyrosine-phosphorylated, suggesting that SH2 domains in SHP-2 are not necessary for recruitment and its tyrosine phosphorylation by JAK2. To further characterize interactions between the SH2 domain deleted Flag-SHP-2 and JAK2, Flag-SHP-2 proteins were immunoprecipitated from cells cotransfected with Flag-SHP-2 and JAK2 using anti-Flag antibodies and analyzed for their tyrosine phosphorylation and association with JAK2. As shown in
reinforce the importance of the linker region between amino acids 232 and 272.

Protein-protein interactions can be mediated by a variety of functional domains such as SH2, SH3, pleckstrin homology, and phosphotyrosine binding domains (29). It is known that SHP-2 SH2 domains interact with tyrosine-phosphorylated receptor tyrosine kinases such as epidermal growth factor receptor tyrosine kinase and other tyrosine-phosphorylated signaling molecules (1, 2, 6, 12–14). Interestingly, our experiments showed that SHP-2 lacking SH2 domains is still tyrosine-phosphorylated by and associated with JAK2 when cotransfected into COS-1 cells (Fig. 3B), demonstrating that SH2 domains in SHP-2 do not interact with JAK2. SHP-2 has been shown to associate with JAK2 in certain lymphoma cells, and the level of JAK2 that associates with SHP-2 in these cells did not change whether JAK2 is tyrosine-phosphorylated and activated (16). Because most of protein-protein interactions mediated by SH2 domains are dependent on protein-tyrosine phosphorylation (29), these data further support our observation that SHP-2 SH2 domains may not be essential for SHP-2 and JAK interactions.

Tyrosine Residues 304 and 327 in SHP-2 Are Phosphorylated by JAKs—As shown in Fig. 2B, SHP-2m1 (amino acids 1–332) and SHP-2m3 (amino acids 1–318), but not SHP-2m4 (amino acids 1–293), were tyrosine-phosphorylated. Sequence comparison between SHP-2m2 and SHP-2m3 indicated that SHP-2m3 contains two tyrosine residues at 304 and 327. C-terminal deletion of tyrosine 327 (construct SHP-2m3) abolished the mobility shift seen in construct SHP-2m2 (Fig. 2B, top panel), suggesting that tyrosine 327 is phosphorylated and responsible for the mobility shift. Because SHP-2m3, but not SHP-2m4, is tyrosine-phosphorylated (Fig. 2B) and the major difference between the two is the presence of tyrosine residue 304 in SHP-2m3, we speculated that residue 304 of SHP-2 may be a potential phosphorylation site for JAKs. To determine whether tyrosine residue 304 is indeed phosphorylated by JAKs, a SHP-2 mutant cDNA containing a point mutation at amino acid 304 from tyrosine to phenylalanine (SHP-2m3*) was constructed and cotransfected with JAK1 or JAK2 into COS-1 cells. As shown in Fig. 4A, mutation of tyrosine 304 to phenylalanine (SHP-2m3*) eliminated tyrosine phosphorylation of the molecule. These results suggest that both tyrosine residues at 304 and 327 are phosphorylated. It was noted that tyrosine at 304 (YINA) contains the preferred sequence motif for SH2 binding by the downstream adapter protein Grb2. We, therefore, examined whether tyrosine-phosphorylated SHP-2m1, SHP-2m2, and SHP-2m3 could bind to Grb2. As shown in Fig. 4B, tyrosine-phosphorylated SHP-2m1, SHP-2m2, and SHP-2m3 from COS-1 cells cotransfected with various SHP-2 mutant and JAK2 cDNAs can be coprecipitated with endogenous Grb2 proteins using anti-Grb2 antibodies. Although previous studies have demonstrated that phosphorylation of tyrosine residue 546 (VTNI) in SHP-2 is essential for mediating SHP-2 and Grb2 interactions (30), our data showed that SHP-2m1 and SHP-2m3, which lack tyrosine residue 546, can still interact with Grb2. In addition, SHP-2m1, which contains amino acids 1–547 including tyrosine residue 546 but not the Grb2 binding motif (YXNX) is still tyrosine-phosphorylated and binds to Grb2 (Fig. 4B), indicating that tyrosine 546 and the surrounding sequences may not be the only motif that Grb2 binds to. These results, therefore, suggest that tyrosine 304 may be another mediator for an alternative pathway coupling SHP-2 to Grb2 signaling.

Identification of JAK2 Domains Required for Association with SHP-2—To characterize which portions of JAK are necessary for SHP-2 and JAK interactions, several deletion mu-
tantsofJAK2(Fig.5A)werealsoconstructed.AsshowninFig.
5B,aninternaldeletionofthekinase-likedomain(aminoacids
521–745) in JAK2 did not affect the association between JAK2
and SHP-2. Deletion of the first 393 amino acids at the N
terminusofJAK2almostcompletelyabrogatedtheassociation
between JAK2 and SHP-2 (a very faint band of JAK2 lacking
the N terminus can be seen in SHP-2 coimmunoprecipitation
after much longer exposure). Further deletion of N-terminal
JAK2 at KpnI site (deleting amino acids 1–719) completely
abolished JAK2 and SHP-2 association. As shown in Fig. 5B,
deletion of the kinase-like domain and the N terminus of JAK2
did not affect JAK2 autophosphorylation and activation in
transfected COS-1 cells. It is clear that JAK2, deleted of its N
terminus, although not able to form stable complexes with
SHP-2, can still phosphorylate SHP-2 in COS-1 cell cotransfec-
tion experiments. This could be due to the weak interactions
between SHP-2 and JAK2 deleted of its N terminus, and the
association could have been disrupted during extraction. Addi-
tional studies using more sensitive methods, such as the yeast
two-hybrid system, will be required to verify such interactions.
Nevertheless, these results suggest that the N-terminal region
of JAK2 is responsible for high affinity association between
JAK2 and SHP-2. Recent studies suggested that the N-termi-
nal JAK homology domain may also interact with cytokine
receptors (31), although precise sequence motifs for such in-
teractions is not yet known. Therefore, further identification of

FIG.4. Phosphorylation of tyrosine 304 in SHP-2 by JAK ki-
nases and its association with Grb2. A, status of tyrosine phospho-
rylation of SHP-2m3 and SHP-2m3*. Tyrosine 304 in SHP-2 was mutated
to phenylalanine by polymerase chain reaction technique and named as
SHP-2m3. SHP-2m3 or SHP-2m3* cDNAs (8 μg) were cotransfected into
cOS-1 cells with 15 μg of JAK1(right panels) or JAK2 (left panels)
cDNA as indicated. Proteins for SHP-2m3 and SHP-2m3* were immuno-
precipitated with anti-SHP-2 antibodies 48 h after transfection. The
precipitated proteins were immobilized on PVDF membranes after sep-
aration with 8% SDS-PAGE. The membranes were immunoblotted with
anti-Tyr(P) (anti-PTyr) antibodies (top), and the same membranes were
reblotted with monoclonal anti-SHP-2 (middle) or anti-JAK (bottom)
antibodies. B, association of different SHP-2 mutants with Grb2. COS-1
cells were cotransfected with 8 μg of different SHP-2 mutant cDNAs
plus 15 μg of JAK2 cDNA as indicated. Grb2 proteins and their asso-
ciated molecules were immunoprecipitated with anti-Grb2 antibodies
48 h after transfection. The precipitated proteins were immobilized on
PVDF membrane after separation with 8% SDS-PAGE. The membrane
was immunoblotted with anti-Tyr(P) (anti-PTyr) antibodies (top) to
visualize tyrosine-phosphorylated proteins coprecipitated with Grb2.
The membrane was then immunoblotted with anti-Grb2 (middle) or
anti-SHP-2 (bottom) as indicated.

FIG.5. N-terminal region of JAK2 is essential for high affinity
association between JAK2 and SHP-2. A, schematic diagram of
JAK2 mutants. All JAK2 mutants were constructed by subcloning
fragments of restriction enzyme digestion as described under “Experi-
mental Procedures” and named as indicated. aa, amino acid(s). B, tyrosine phosphorylation and association of SHP-2 with different JAK2
mutants. COS-1 cells were transfected with wild-type SHP-2 (8 μg) plus
different JAK2 mutant cDNAs (15 μg) as indicated. The same cell
lysates were first immunoprecipitated with anti-SHP-2 antibodies (left
panels) and then with anti-JAK2 (right panels) antibodies as indicated.
The precipitated proteins were immobilized on PVDF membranes after
separation with 8% SDS-PAGE. The membranes were immunoblotted
with anti-Tyr(P) (anti-PTyr) antibodies (top) and then with anti-SHP-2
or JAK2 antibodies (bottom) as indicated.
specific sequence motifs in the N-terminal JAK homology domain is required to fully understand physiological roles of JAK and SHP-2 interactions.

In summary, we have demonstrated that SHP-2 is a substrate for JAK1 and JAK2. Deletion studies indicated that the sequences between amino acids 232–272, but not the SH2 domains of SHP-2, are required for JAK and SHP-2 interactions. Furthermore, tyrosine residues 304 and 327 in the SH2 domain are phosphorylated by JAK kinases, and phosphorylated tyrosine 304 is capable of binding to Grb2. We have also shown that the N-terminal JAK homology domain is important for high affinity association between JAK and SHP-2. Our data indicate that novel sequences in SHP-2 are involved in the association between SHP-2 and JAKs, which differs from the interactions between SHP-2 and various receptor tyrosine kinases that are thought to be mediated by SHP-2 SH2 domains. These findings implicate novel mechanisms in protein-protein interactions and potential physiological roles between JAK and SHP-2 interactions in cellular signaling.

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