MOLECULAR AND CELLULAR BIOLOGY, Dec. 1996, p. 6985–6992 Vol. 16, No. 12
0270-7306/96/$04.00+0

Direct Association with and Dephosphorylation of Jak2 Kinase by the SH2-Domain-Containing Protein Tyrosine Phosphatase SHP-1

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Received 26 July 1996/Returned for modification 28 August 1996/Accepted 17 September 1996

SHP-1 is an SH2-containing cytoplasmic tyrosine phosphatase that is widely distributed in cells of the hematopoietic system. SHP-1 plays an important role in the signal transduction of many cytokine receptors, including the receptor for erythropoietin, by associating via its SH2 domains to the receptors and dephosphorylating key substrates. Recent studies have suggested that SHP-1 regulates the function of Jak family tyrosine kinases, as shown by its constitutive association with the Tyk2 kinase and the hyperphosphorylation of Jak kinases in the motheaten cells that lack functional SHP-1. We have examined the interactions of SHP-1 with two tyrosine kinases activated during engagement of the erythropoietin receptor, the Janus family kinase Jak-2 and the c-fps/fes kinase. Immunoblotting studies with extracts from mouse hematopoietic cells demonstrated that Jak2, but not c-fes, was present in anti-SHP-1 immunoprecipitates, suggesting that SHP-1 selectively associates with Jak2 in vivo. Consistent with this, when SHP-1 was coexpressed with these kinases in Cos-7 cells, it associated with and dephosphorylated Jak2 but not c-fes. Transient cotransfection of truncated forms of SHP-1 with Jak2 demonstrated that the SHP-1–Jak2 interaction is direct and is mediated by a novel binding activity present in the N terminus of SHP-1, independently of SH2 domain-phosphotyrosine interaction. Such SHP-1–Jak2 interaction resulted in induction of the enzymatic activity of the phosphatase in in vitro protein tyrosine phosphatase assays. Interestingly, association of the SH2n domain of SHP-1 with the tyrosine phosphorylated erythropoietin receptor modestly potentiated but was not essential for SHP-1-mediated dephosphorylation of Jak2 and had no effect on c-fes phosphorylation. These data indicate that the main mechanism for regulation of Jak2 phosphorylation by SHP-1 involves a direct, SH2-independent interaction with Jak2 and suggest the existence of similar mechanisms for other members of the Jak family of kinases. They also suggest that such interactions may provide one of the mechanisms that control SHP-1 substrate specificity.

Protein tyrosine phosphorylation is a common mechanism of signal transduction and is controlled by the balance of protein tyrosine kinases (PTKases) and protein tyrosine phosphatases (PTPases) (11). SHP-1 (1) is a PTPase predominantly expressed in cells of the hematopoietic system; it is also termed PTP1C, HCP, SHPTP1, and SHP (15, 21, 24, 44). Loss of functional SHP-1 because of mutations in the mouse SHP-1 gene is associated with motheaten disease, characterized by increased phosphorylation in hematopoietic cells, hypersensitivity to extracellular stimuli, and heightened myelopoiesis (25, 26, 32). This indicates that SHP-1 is a critical negative regulator of tyrosine phosphorylation and signal transduction in hematopoietic cells.

SHP-1 is a cytoplasmic protein with two src-homology 2 (SH2) domains at the amino region (SH2n and SH2c) and a PTPase catalytic domain at the carboxyl terminus (44). SHP-1 may therefore regulate protein tyrosine phosphorylation in hematopoietic cells by associating with tyrosine-phosphorylated proteins through its SH2 domains and by dephosphorylating tyrosine-phosphorylated proteins through its phosphatase domain. Identification of the phosphotyrosine proteins interacting with SHP-1 is essential for understanding the function of this phosphatase in hematopoietic cells.

Several phosphotyrosine proteins that specifically interact with SHP-1 SH2 domains have been reported, and they are all cell membrane receptors. We have shown that SHP-1 binds to the receptor for stem cell factor (c-kit), interleukin-3 receptor (IL-3R), and erythropoietin receptor (EpoR) following ligand-binding-induced receptor tyrosine phosphorylation (14, 40–42). Overexpression of SHP-1 results in the down-regulation of interleukin-3-induced protein tyrosine phosphorylation and cell proliferation (41), indicating that SHP-1 couples with cell surface receptors to down-regulate tyrosine phosphorylation and mitogenic signaling initiated by hematopoietic growth factors. This is consistent with the growth factor hypersensitivity (13, 33) and the overproduction of hematopoietic cells in the motheaten mice (25). Recent studies have demonstrated that SHP-1 also associates with other membrane phosphotyrosine proteins: the B-cell receptor for immunoglobulin G Fc domains (FcgRIIB1), the CD22 molecule, and the natural killer (NK) cell inhibitory receptor p58 (3, 5, 10). SHP-1 binds to FcgRIIB1 and CD22, which associate with the membrane immunoglobulin antigen receptor (BCR) on B lymphocytes, and down-regulates signals initiated from BCR (5, 10). A negative regulatory role of SHP-1 in BCR signaling is also supported by the observation that the B cells from motheaten mice are

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hypersensitive to antigen stimulation (4). The SHP-1-coupled p58 functions as the receptor on NK cells for major histocompatibility complex class I molecules (3). Since ligand binding to p58 inhibits NK cells from lysing target cells, the inhibitory effect of p58 may be mediated by SHP-1, which down-regulates the signal required for NK cell killing. The two SH2 domains of SHP-1 target different receptors: the association of SHP-1 to the receptors of hematopoietic growth factors is mediated via the SH2n domain (3, 14, 40–42), while the SH2c domain is responsible for SHP-1 binding to the other membrane signaling receptors (3, 5, 10). Several phosphotyrosine sites in the cytoplasmic region of these receptors were found to be potential docking sites for the SHP-1 SH2 domains, and their binding to SHP-1 SH2 domains leads to an increase in SHP-1 PTPase activity (3, 5, 10, 14, 18, 42). This indicates that the SH2 domain region regulates the PTPase catalytic activity, and it is consistent with the observation that the deletion of the amino region of SHP-1 markedly activates the phosphatase (19, 31).

Few substrates that are dephosphorylated by SHP-1 in vivo have been identified. The association of SHP-1 with the phosphotyrosine membrane receptors brings it in close contact with cellular phosphotyrosine proteins that associate with or are in the vicinity of the receptors. For the EpoR, these include Jak2 (37), c-fps/fes (12), PI3K (6), SHC (7), STAT5 (8), Syp (30), and Vav (17). Given the crucial role for SHP-1 in regulating signal transduction, additional mechanisms to control SHP-1 substrate specificity may exist. Several lines of evidence indicate that the Jak family of kinases may be substrates regulated by SHP-1. SHP-1 was shown to down-regulate the tyrosine phosphorylation and cell proliferation induced by interleukin-3, which depends on Jak2 for signaling (41). Moreover, Jak kinases in macrophages from motheaten mice lacking functional SHP-1 were shown to be hyperphosphorylated following alpha/beta interferon-treatment (9).

Our recent study demonstrated that granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of motheaten macrophages induces a modest and transient Jak2 hyperphosphorylation (13), consistent with a role for SHP-1 in down-regulating Jak2 phosphorylation. While it was not clear whether SHP-1 down-regulation of Jak2 phosphorylation was mediated by a direct or an indirect mechanism and whether it requires the participation of additional molecules, our recent finding that SHP-1 forms preexisting complexes with Tyk2 suggests a direct interaction between SHP-1 and the Jak family kinases (39). However, conflicting results have been reported regarding the role of the SH2 domain of SHP-1 in mediating dephosphorylation of target substrates. One study has shown that mutation of two potential SHP-1 docking sites in the EpoR causes marked Jak2 hyperphosphorylation in response to Epo stimulation (14). Another study, however, demonstrated that Jak2 phosphorylation induced by engagement of an EpoR null mutant with each of the tyrosines in the receptor cytoplasmic domain substituted by phenylalanine was comparable to those induced through the wild-type EpoR (8). In the present study, we examined the interaction of SHP-1 with two cytoplasmic PTKases activated in Epo signaling, Jak2 and c-fps/fes, in the absence or presence of tyrosine-phosphorylated EpoR. We found that SHP-1 associates directly with Jak2 but not c-fes through a novel mechanism, independently of SH2 domain-phosphotyrosine interaction. This association leads to SHP-1 activation and dephosphorylation of Jak2 and may serve to control SHP-1 substrate specificity.

MATERIALS AND METHODS

Reagents. cDNA clones for murine c-fgr (45), lyn (43), EpoR (37), and SHP-1 (44) have been described previously. The murine Jak1 (34), Jak2 (27), Jak3 (36), and c-fes (35) cDNA clones were generated by PCR from a murine hematopoietic cell cDNA library (45) with primers based on published sequences. The human Tyk2 cDNA was a gift from George Stark. The murine SHP-1 cDNA K30, K136, and S453 mutants and the Jak2/E882 mutant were generated by site-directed mutagenesis as described previously (41) and sequenced by a single-chain termination method (23) to confirm the correct mutations. The truncational mutants of the N region (amino acids [aa] 1 to 267), C region (aa 247 to 595), N1 (aa 1 to 106), and N2 (aa 107 to 267) were generated by PCR from the null mutant of SHP-1. A translation initiation codon (ATG) was incorporated at the beginning of the mutants. The cDNA clones were inserted into the eukaryotic cell expression vector pRK5 or pXM as indicated.

The SHP-1, SHP-1 mutants, Jak2, and c-fes cDNAs were also cloned into the pGEX vector, expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*, purified by glutathione affinity chromatography as described previously (40, 41), and used in PTPase assays (3) and binding assays (40).

The antibodies against SHP-1, EpoR, lyn, and c-fes have been reported previously (37, 44). Antisera against Jak kinases and monoclonal antibody (4G10) specific for phosphotyrosine were purchased from UBI. Recombinant Epo was from Amgen (Thousand Oaks, Calif.).

Cells, cell culture, transient transfection, and cell stimulation. Spleens were dissected from C57BL/6J normal (+/+) or viable motheaten (*mev/mev*) mice and used to prepare cell suspensions in RPMI-1640 medium supplemented with 10% fetal calf serum. Cos-7 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Cos-7 cells were transiently transfected with different constructs by a DEAE-dextran method (42). The transfected cells were stimulated with recombinant Epo (3 U/ml) at 37°C for various times as indicated. The stimulations were terminated by lysing the cells in lysis buffer (50 mM Tris [pH 7.4], 50 mM NaCl, 0.5% sodium deoxycholate, 0.2 mM Na_3VO_4, 20 mM NaF, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 10% glycerol).

Immunoprecipitations and Western blotting (immunoblotting) (43). Cells were lysed in cold lysis buffer. Cell lysates were clarified by centrifugation for 20 min at 10,000 × *g* at 4°C. Antisera were added to cell lysates and incubated at 4°C for 60 min with gentle agitation. Immune complexes were collected with protein A Sepharose 4B (Pharmacia) at 4°C for 30 min, washed gently in cold lysis buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting, protein samples separated on SDS-PAGE gels were blotted onto nitrocellulose membrane (Schleicher & Schuell). The membranes were then blocked with 5% milk in washing buffer (10 mM Tris, [pH 7.4], 150 mM NaCl, 0.1% Tween 20) for 2 h, incubated with rabbit polyclonal antibodies (1:5,000 dilution of whole serum) or with the mouse monoclonal anti-phosphotyrosine antibody 4G10 (1 μg/ml; UBI) for 2 h, washed for 1 h, incubated with secondary antibodies for 1 h, and washed for another hour. Specific antibody signals were detected with an enhanced chemiluminescence kit (ECL; Amersham).

In vitro binding assay and PTPase assay. The preparation of GST fusion proteins has been described previously (40). For binding assays, a GST fusion protein of the N region of SHP-1 (0.1 μg) was incubated with GST-Jak2 (0.4 μg) at 4°C for 12 h and washed 3 times in lysis buffer. Protein complexes were collected at 4°C for 1 h with protein A Sepharose 4B beads precoated with an anti-SHP-1 antibody and were analyzed by SDS-PAGE and Western blotting with an anti-Jak2 antibody. The PTPase activity of SHP-1 fusion proteins was determined using pNPP (Sigma) as substrate (3). The PTPase assay was carried out at 37°C for 30 min in 100 μl of reaction mixture (100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM pNPP, 0.240 μM peptides, 20 nM fusion protein) with 1 mM GST–SHP-1 in the absence or presence of 10 mM GST-Jak2 or GST–c-fes. PTPase activity of 10 mM GST-Jak2 or GST–c-fes was also determined under the same conditions as a control. The reaction was terminated by the addition of 900 μl of 1 N NaOH. The PTPase reaction product *p*-nitrophenolate was quantitated by measuring A_{405}.

RESULTS

Jak2 coimmunoprecipitates with SHP-1 from murine spleen cells. In order to detect potential interactions of SHP-1 with the Jak2 kinase in hematopoietic cells, we first immunoprecipitated SHP-1 from the spleen cells of normal (+/+) or viable motheaten (*mev/mev*) mice and probed the immunocomplexes with an anti-Jak2 antibody. Jak2 was detected in the anti-SHP-1 immunocomplexes from both normal (Fig. 1, lane 1) and motheaten (lane 2) cells, while there was a modest increase in the amount of Jak2 coimmunoprecipitated with the two mutated forms of SHP-1 from the motheaten cells. On the other hand, we failed to detect c-fes in the immunocomplexes

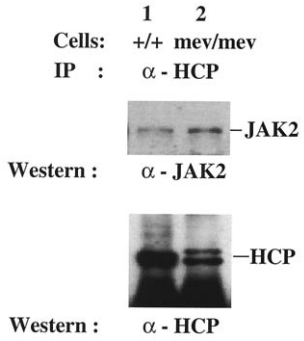

FIG. 1. Detection of Jak2 kinase in anti-SHP-1 immunoprecipitates. Cell lysates were prepared from the spleen cells of normal (+/+) or the viable motheaten (*mev*/*mev*) mice and incubated with an anti-SHP-1 antibody. The immunocomplexes were analyzed by SDS-PAGE and Western blotting with antibodies as indicated. The two differently sized SHP-1 proteins in the *mev*/*mev* sample resulted from the *mev* mutations (26).

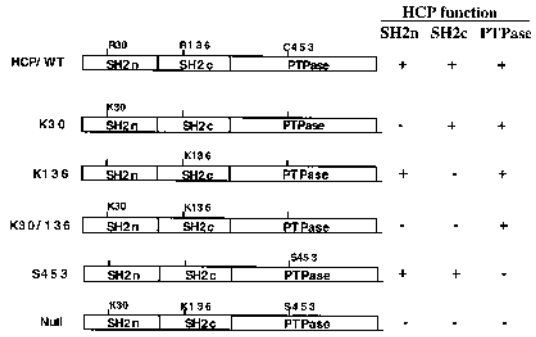

FIG. 3. Structure and functions of SHP-1 and SHP-1 mutants. The conserved arginine (R30 and R136) in the SH2 domains and the cysteine (C453) in the PTPase domain of SHP-1 are required for the SH2 domain binding and the PTPase activities, respectively (8, 40). These residues were substituted with lysines (K30 and K136) and serine (S453) by site-specific mutagenesis of the murine SHP-1 cDNA as indicated. The functions of the SHP-1 mutants were determined by phosphotyrosine protein binding assays (40) and PTPase assays (3). Results are summarized on the right.

(data not shown). These results suggested that SHP-1 may form preexisting complexes with Jak2 to regulate Jak phosphorylation and function. However, it was not clear whether SHP-1 associates with the kinase via a direct mechanism or through receptors coupled with Jak2.

SHP-1 associates with and dephosphorylates Jak2 but not c-fes when coexpressed in Cos-7 cells. To determine whether SHP-1 directly interacts with and dephosphorylates Jak2, we coexpressed SHP-1 and Jak2 in Cos-7 cells by transient transfection. Cos-7 cells did not express endogenous SHP-1 (Fig. 2D, lanes 1 and 2) as expected. The endogenous Jak2 kinase in Cos-7 cells was not tyrosine phosphorylated (Fig. 2A, lane 1). Consistent with results of a previous report (38), Jak2 from Cos-7 cells transfected with the murine Jak2 construct was strongly tyrosine phosphorylated (Fig. 2A, lane 2). Cotransfection of SHP-1 with Jak2 resulted in their coimmunoprecipitation (Fig. 2C, lane 3) and a substantial reduction in Jak2 tyrosine phosphorylation (Fig. 2A, lane 3), indicating that SHP-1 directly associated with and dephosphorylated Jak2 in vivo. In contrast, SHP-1 had no significant effect on the tyrosine phosphorylation of c-fes when coexpressed in Cos-7 cells (Fig. 2A, lane 6), and we failed to detect c-fes coimmunoprecipitation with SHP-1 under comparable conditions (Fig. 2C, lane 6).

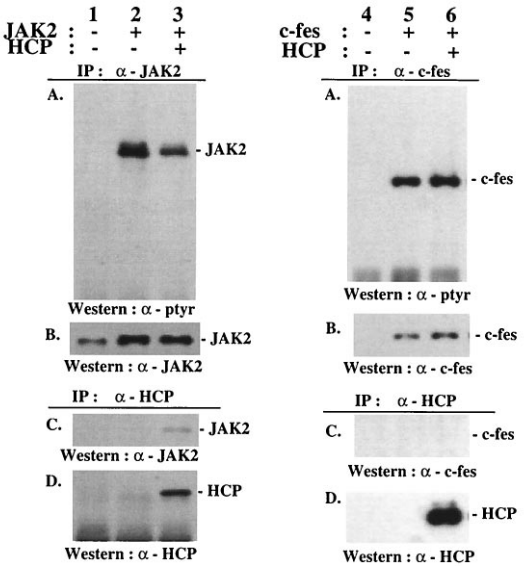

FIG. 2. SHP-1 associates with and dephosphorylates Jak2 but not c-fes when coexpressed in Cos-7 cells. Cos-7 cells were transiently transfected with the vector PRK5 alone (lanes 1 and 4) or PRK5 expression constructs of murine Jak2, SHP-1, or c-fes as indicated. Cell lysates were prepared from the transfectants and used for immunoprecipitation. Immunocomplexes were analyzed by SDS-PAGE and Western blotting with antibodies as indicated. The positions of Jak2, c-fes, and SHP-1 are indicated.

SHP-1 binds to and dephosphorylates Jak2 in the absence of functional SH2 domains. To define the role of the functional domains of SHP-1 in Jak2 interaction and dephosphorylation, we generated a series of murine SHP-1 mutants with single amino acid substitutions that selectively affect the function of the SH2n, SH2c, or PTPase domain of the phosphatase (Fig. 3). R30, R136, and C453 are conserved amino acid residues required for the functions of the SH2n, SH2c, and PTPase domains, respectively. The K30 and K136 mutants in SHP-1 have been shown to abolish SH2n and SH2c binding to phosphotyrosine substrates, respectively (40–42), while SHP-1 mutants containing the S453 mutation showed no PTPase activity (3).

Our initial experiments showed that all the growth factor-dependent hematopoietic cell lines examined express SHP-1 at different levels, thus making them inappropriate for analysis of these SHP-1 mutants, as the endogenous SHP-1 would complicate data interpretation. Nonhematopoietic cell lines such as NIH3T3 and Cos-7 cells do not express SHP-1. But in these cells, we failed to establish stable transfectants of SHP-1 or SHP-1 mutants, including the null mutant (data not shown). We therefore coexpressed SHP-1 and the SHP-1 mutants with Jak2 in Cos-7 cells by transient transfection and examined their interaction with the kinase and their effect on Jak2 phosphorylation.

Jak2 was associated with each of the SHP-1 mutants that lack the functions of the SH2 domains or PTPase domain (Fig. 4B, lanes 2 to 6). The amount of Jak2 in association with the SHP-1 mutants (except the K136 mutant) (Fig. 4B, lanes 2, 4, 5, and 6) was one- to threefold higher than that of the wild-type SHP-1 (lane 1). This suggests that SHP-1 binds to Jak2 via a novel mechanism not requiring its known functional domains and that mutations in the SH2n and PTPase domains may further facilitate or stabilize SHP-1–Jak2 interaction. The Jak2 coimmunoprecipitated with each of the SHP-1 mutants with a functional PTPase domain was not tyrosine phosphorylated

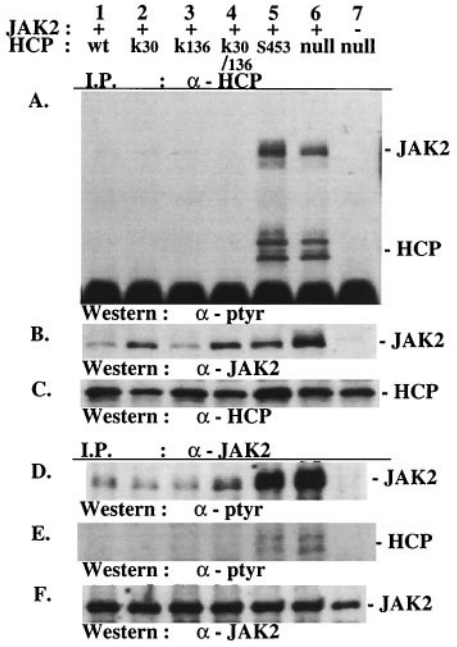

FIG. 4. SHP-1 directly associates and dephosphorylates Jak2 independently of the function of its SH2 domains. Expression constructs of murine SHP-1 and SHP-1 mutants or murine Jak2 in different combinations as indicated were coexpressed in Cos-7 cells by transient cotransfection (42). SHP-1 and Jak2 proteins were immunoprecipitated separately from prepared cell lysates with corresponding antibodies and analyzed by SDS-PAGE and Western blotting with specific antibodies as indicated. The positions of Jak2 and SHP-1 are indicated.

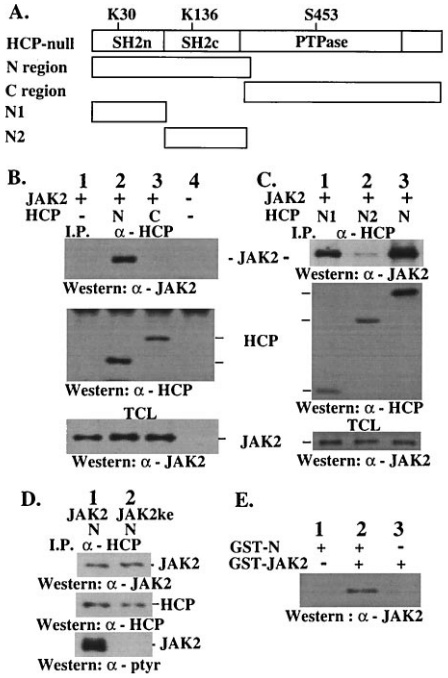

FIG. 5. The amino-terminal region of SHP-1 binds directly to Jak2 kinase. (A through D) The amino region (N), carboxyl region (C), and N1 and N2 portions of the SHP-1 null mutant in the pRK5 vector were coexpressed with pRK5-Jak2 or pRK5-Jak2ke in Cos-7 cells by transient transfection. Cell lysates were prepared and incubated with an anti-SHP-1 antibody. Immunocomplexes were analyzed by SDS-PAGE and Western blotting with antibodies as indicated. (E) GST fusion proteins of the N region of SHP-1 and Jak2 were incubated to allow complex formation. Protein complexes were immunoprecipitated with an anti-HCP antibody and analyzed by SDS-PAGE and Western blotting with an anti-Jak2 antibody. The positions of Jak2 and SHP-1 are indicated.

(Fig. 4A, lanes 1 to 4), providing strong evidence that SHP-1 directly dephosphorylates Jak2 in vivo. It also showed that SHP-1 dephosphorylated Jak2 independently of the functions of its SH2 domains, as association of Jak2 with the SHP-1 K30/K136 mutant also resulted in dephosphorylation of the kinase (lanes 2 to 4). Similar, but incomplete, dephosphorylation of Jak2 was also observed when anti-Jak2 immunocomplexes were examined (Fig. 4D). This incomplete dephosphorylation may result from some of the Jak2 that was not in association with the phosphatase in the cells. As reported previously (2), SHP-1 mutants lacking PTPase activity (lanes 5 and 6) were tyrosine phosphorylated. These hyperphosphorylated SHP-1 mutants associated with Jak2 (Fig. 4A through E, lanes 5 and 6) and migrated as multiple bands, probably as a result of differential phosphorylation. In addition, SHP-1 and its mutants showed similar association and dephosphorylation of the other Jak family members (JAK1, Tyk2, and JAK3) but not c-fes when coexpressed in Cos-7 cells (data not shown).

The amino region of SHP-1 mediates its binding to Jak2, independently of SH2 domain-phosphotyrosine interaction. To determine the location of the Jak2 binding activity in SHP-1, we generated expression constructs containing the amino region (aa 1 to 267) or the carboxyl region (aa 247 to 595) of the SHP-1 null mutant (Fig. 5A), and cotransfected them with Jak2 in Cos-7 cells. Jak2 was coimmunoprecipitated with the SHP-1 amino region (N) (Fig. 5B, lane 2) but not the carboxyl region (C) (lane 3). The N region also bound to a Jak2 mutant (JAK2E882) that was not tyrosine phosphorylated (Fig. 5D) as a result of a substitution mutation of Lys-882 by Glu that inactivates the kinase (22). Moreover, a GST fusion protein containing the N region bound to a GST-Jak2 fusion protein in vitro (Fig. 5E). These data demonstrated that the Jak2 binding activity in SHP-1 is located at the amino region and is not dependent on SH2 domain-phosphotyrosine interactions or additional molecules. Since this amino region also associated with all the other members of the Jak family (JAK1, Tyk2, and JAK3) but not c-fes under comparable conditions (data not shown), this specific binding activity may recognize a common target site that is conserved among the members of the Jak kinase family.

To further define the location of the binding activity, we divided the N region into two portions (N1 and N2) (Fig. 5A), coexpressed them with Jak2 in Cos-7 cells, and probed the N1 and N2 immunocomplexes with an anti-Jak2 antibody. Jak2 was detected primarily in the immunocomplex of the N1 portion (aa 1 to 106) (Fig. 5C, lane 1) with a weak Jak2 signal associated with the N2 portion (aa 107 to 267) (Fig. 5C, lane 2). These results indicated that the Jak2 binding activity of SHP-1 is located predominantly in the N1 portion.

SHP-1 PTPase activity is activated by Jak2. SHP-1 PTPase activity is up-regulated by peptides binding to the SH2 domains at the amino region (3, 5, 10, 18). The demonstrated binding of SHP-1 via its amino region to Jak2 suggested that this interaction may affect SHP-1 PTPase activity. We therefore determined the PTPase activity of a GST fusion protein of the SHP-1 K30/K136 mutant in the absence or presence of a GST-Jak2 or GST–c-fes fusion protein. As shown in Fig. 6, SHP-1 PTPase activity was substantially increased in the presence of Jak2 but not c-fes. GST-Jak2 or GST–c-fes alone had little PTPase activity (data not shown). Thus the Jak2–SHP-1 interaction appears to activate the catalytic activity of SHP-1. Since the PTPase assays were performed in the absence of ATP,

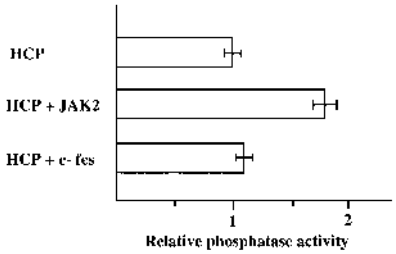

FIG. 6. Jak2 activates SHP-1 PTPase activity. The PTPase activity of a GST–SHP-1 fusion protein with K30/K136 mutations was determined in the absence or presence of GST-Jak2 or GST–c-fes as indicated. The data represent the mean ± standard deviation values of triplicate samples.

which is required for Jak2 autophosphorylation or the phosphorylation of SHP-1 by the Jak2 kinase, these data indicate that the activation of SHP-1 by Jak2 is independent of Jak2 kinase activity.

Binding of SHP-1 via its SH2 domains to EpoR modestly potentiates SHP-1 dephosphorylation of Jak2. SHP-1 associates via its SH2n domain with the tyrosine-phosphorylated EpoR and down-regulates EpoR signaling (14, 42). Both Jak2 (37) and c-fes (12) have been implicated in EpoR signaling. To determine the effect of SHP-1 SH2n binding to EpoR on SHP-1–Jak2 or SHP-1–c-fes interaction and dephosphorylation, we coexpressed EpoR–Jak2–SHP-1 or EpoR–c-fes–SHP-1 in Cos-7 cells.

EpoR expressed alone or with Tyk2, JAK1, JAK3, c-fgr, or lyn kinase in Cos-7 cells was not significantly tyrosine phosphorylated without or with Epo stimulation (data not shown). The coexpression of EpoR and Jak2 resulted in the tyrosine phosphorylation of both proteins, and their phosphorylation was inducible by Epo stimulation (Fig. 7, lanes 1 and 2). The phosphorylation of the receptor and the kinase was markedly down-regulated (5- to 10-fold) in the presence of SHP-1 (Fig. 7A and B, lanes 3 and 4) as well as the SHP-1 K30/136 mutant (Fig. 7A and B, lanes 7 and 8), further establishing the fact that functional SH2 domains are not essential for Jak2 dephosphorylation by SHP-1. The phosphotyrosine content of Jak2 coexpressed with the SHP-1 K30/136 mutant was about onefold higher than that of the Jak2 coexpressed with SHP-1 (compare Fig. 7C and D, lanes 3 and 4 and 7 and 8). This indicated that the mutant lacking functional SH2 domains was slightly less efficient than the wild-type SHP-1 in dephosphorylating Jak2, suggesting that binding of the SHP-1 SH2 domain to the EpoR only modestly facilitates the process.

Interestingly, coexpression of S453 mutant correlated with a marked increase in EpoR tyrosine phosphorylation (Fig. 7A, lanes 5 and 6). The presence of functional SH2 domains in the mutant indicates that the increase in EpoR phosphorylation likely resulted from the binding of the SHP-1 SH2 domains to phosphotyrosines in the EpoR and that occupation of these tyrosines might have prevented dephosphorylation of the receptor. On the other hand, the S453 mutant had no such protective effect on Jak2 phosphorylation (Fig. 7C, lanes 5 and 6), consistent with a lack of direct interaction between the SHP-1 SH2 domains and phosphotyrosine sites in Jak2. Coexpressing the null mutant also had no marked effect on Jak2 phosphorylation (Fig. 7C, lanes 9 and 10), but it was associated with a decrease in EpoR tyrosine phosphorylation (Fig. 7A, lanes 9 and 10; compare lanes 1 and 2). Whether this was the result of binding of the null mutant to Jak2 and thus interfering with the receptor phosphorylation by the kinase remains to be determined. The amount of Jak2 coimmunoprecipitated with SHP-1 and the SHP-1 mutants (Fig. 7G) and their phosphorylation levels (Fig. 7E) were similar to those detected in cells expressing both SHP-1 and Jak2 constructs in the absence of

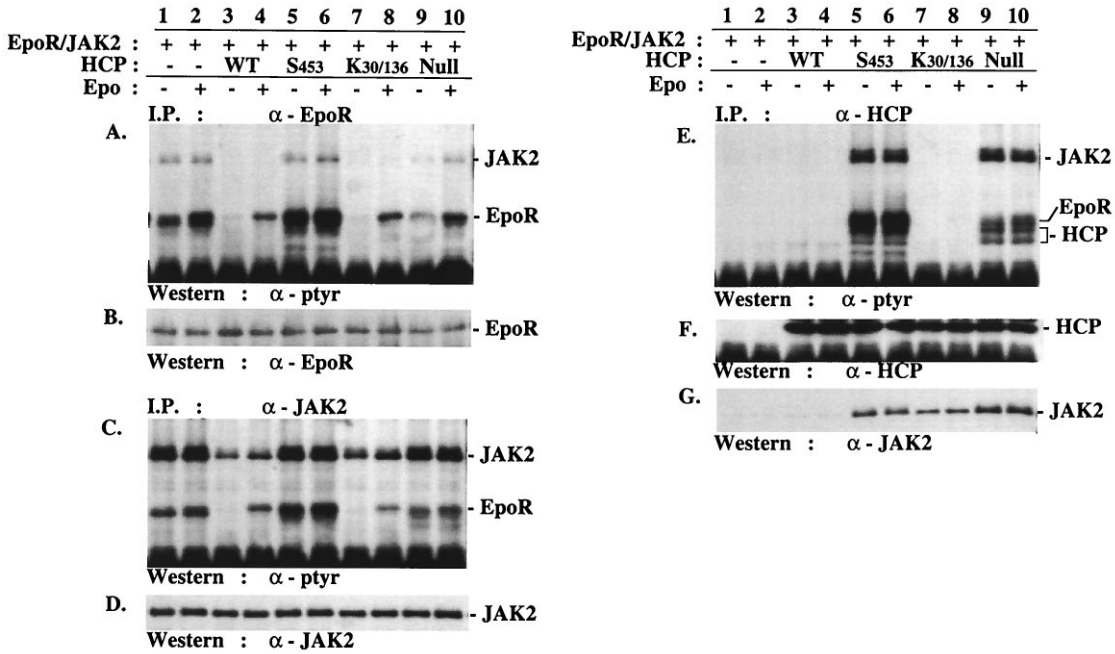

FIG. 7. SHP-1 down-regulates EpoR-Jak2 phosphorylation independently of SHP-1 SH2n binding to EpoR. SHP-1 and SHP-1 mutants were coexpressed with EpoR-Jak2 in Cos-7 cells by transient transfection. The cells were treated without (−) or with (+) Epo for 5 min and used for cell lysate preparation. EpoR (A and B), Jak2 (C and D), or SHP-1 (E to G) was immunoprecipitated and analyzed by SDS-PAGE and Western blotting with antibodies as indicated. The positions of EpoR, Jak2, and SHP-1 are indicated.

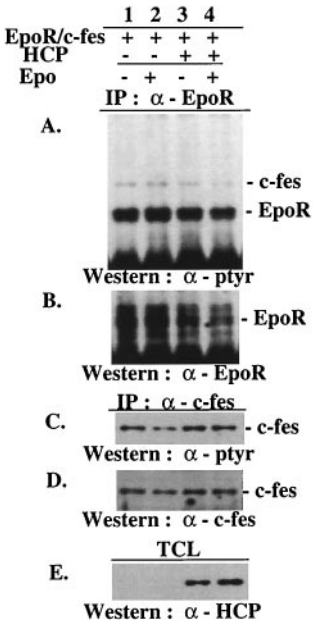

FIG. 8. The tyrosine phosphorylation of c-fes–EpoR is not affected by SHP-1. pXM-EpoR and PRK5–c-fes expression constructs were coexpressed with SHP-1 (lanes 3 and 4) in Cos-7 cells by transient transfection. The cells were treated with Epo for 5 min and used for cell lysate preparation. EpoR and c-fes were immunoprecipitated separately and analyzed by SDS-PAGE and Western blotting with antibodies as indicated. The positions of EpoR and c-fes are indicated.

the EpoR (Fig. 4). The amount of Jak2 coimmunoprecipitated with the wild-type HCP was lower (Fig. 7G, lanes 3 and 4) and prolonged exposure of the blot was required for its visualization.

Coexpression of the EpoR with c-fes resulted in a constitutive EpoR and c-fes tyrosine phosphorylation that was not affected by either Epo stimulation (Fig. 8, lanes 1 and 2) or the presence of SHP-1 (lanes 3 and 4). This was not due to failure of the c-fes-phosphorylated EpoR to provide docking sites for SHP-1, because a GST fusion protein containing the SHP-1 SH2 domains bound to the EpoR phosphorylated by either c-fes or Jak2 (Fig. 9). Like Jak2, c-fes was coimmunoprecipitated with the EpoR when coexpressed in Cos-7 cells (Fig. 8A).

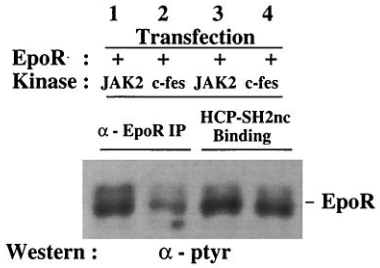

FIG. 9. SHP-1 SH2n domain bound to EpoR phosphorylated by Jak2 or c-fes. EpoR was coexpressed with Jak2 or c-fes in Cos-7 cells by transient transfection. The cells were treated with Epo for 5 min and harvested for lysate preparation. Cell lysates were boiled in 1× SDS-PAGE sample buffer, diluted with 10 vol of lysis buffer, and incubated with an anti-EpoR antibody or a GST fusion protein of the SHP-1 SH2n domain. The immunocomplexes and cellular proteins associated with the fusion protein were analyzed by SDS-PAGE and Western blotting with an antibody against phosphotyrosine.

DISCUSSION

SHP-1 plays a pivotal role in regulating signal transduction in hematopoietic cells by dephosphorylating key substrates, but the precise mechanisms by which it mediates its actions are unknown. The present study provides evidence that SHP-1 directly dephosphorylates the Jak2 kinase in vivo. Our data also demonstrate that the SHP-1 activity selectively regulates Jak2 and other Jak family members but not another cytoplasmic tyrosine kinase, c-fes. The only other tyrosine phosphatase with SH2 domains, Syp, was not effective against Jak2 phosphorylation under comparable conditions (our unpublished data), further demonstrating the specificity of the SHP-1–Jak2 interaction. We also provide evidence that SHP-1 binds directly to the Jak2 kinase. This binding is mediated by a novel activity which is independent of SH2-phosphotyrosine interaction and is located primarily at the amino-terminal 106 amino acids of the phosphatase. The fact that SHP-1 binds to all Jak family members but not other cytoplasmic tyrosine kinases, such as c-fes and lyn (our unpublished data), indicates a common SHP-1 binding site conserved in all Jak kinases.

A intriguing finding from this study is that SHP-1 mutants with the K30 mutation showed increased Jak2 binding. The arginine at position 30 (R30) in SHP-1 is a conserved amino acid in the FLVRE motif, essential for SH2 domain binding activity to phosphotyrosine (16). Substituting a lysine (K30) for the arginine abolishes the in vitro binding of SHP-1 SH2nc GST fusion protein to several tyrosine phosphorylated proteins (40–42). Our finding suggests that there may be a competitive balance between SHP-1 binding to the Jak family kinases or to other phosphotyrosine proteins. Abolishing SH2n function by a K30 mutation may thus increase SHP-1 N region binding to the Jak family kinases. However, no endogenous Cos-7 phosphotyrosine proteins were detected in association with SHP-1 or SHP-1 mutants containing functional SH2 domains (Fig. 4A). Because a mutation (S453) in the SHP-1 PTPase catalytic domain also led to increased Jak2 binding, another explanation is that the mutations may have caused a configurational change in the phosphatase and exposed a cryptic Jak2 binding site. The existence of an additional functional domain(s) besides the known SH2 and PTPase domains in SHP-1 is consistent with our finding that the SHP-1 null mutant could not be stably expressed in any of the cell lines we have tried.

Our finding that SHP-1 directly binds and dephosphorylates JAKs but demonstrates no such activities against c-fes indicates a correlation between binding and dephosphorylation and suggests that SHP-1 binding may be required for substrate targeting. It also provides a potentially important mechanism for down-regulating the kinase phosphorylation without a requirement for SHP-1 binding to phosphotyrosine receptors. The granulocytes of the motheaten mice lacking functional SHP-1 are hypersensitive to G-CSF, which depends on Jak2 for mitogenic signaling, but SHP-1 does bind to the tyrosine phosphorylated G-CSF receptor (29). Our finding that SHP-1 directly dephosphorylates Jak2 may help explain the heightened G-CSF response in the motheaten cells. Importantly, it also provides an explanation for the down-regulation of Jak2 tyrosine phosphorylation induced by engagement of an EpoR null mutant with each of the tyrosines in the receptor cytoplasmic domain substituted by phenylalanine (8). Interestingly, it was reported recently that SHP-1 directly associates with and dephosphorylates ZAP-70 kinase involved in T-cell antigen receptor signaling and that SHP-1 is activated as a result of binding to ZAP-70 (20). Taken together with our data, this suggests that direct substrate binding and PTPase activation

may be common mechanisms of SHP-1–substrate dephosphorylation.

Our data show that both Jak2 and c-fes associate with the EpoR and phosphorylate the receptor when coexpressed in Cos-7 cells. Although the EpoR phosphorylated by either Jak2 or c-fes contains a binding site(s) for the SHP-1 SH2n domain, SHP-1 dephosphorylated Jak2 but not c-fes in the presence of the phosphorylated receptor. Thus SHP-1 recruited to the EpoR via SH2-phosphotyrosine binding is highly specific in dephosphorylating substrates, and the coexistence of a phosphotyrosine protein with the phosphatase in the receptor complex is not sufficient for its dephosphorylation. This suggests the existence of an additional mechanism(s), such as direct SHP-1–substrate binding, controlling SHP-1 substrate specificity. Although binding of SHP-1 to EpoR is not essential for Jak2 dephosphorylation, it may potentiate the process, as an SHP-1 mutant lacking functional SH2 domains was less efficient than the wild-type SHP-1 in dephosphorylating the kinase. This is consistent with a previous report (14) demonstrating that an EpoR mutant lacking potential docking sites for the SHP-1 SH2n domain induced prolonged hyperphosphorylation of Jak2. However, additional mechanisms may be involved in causing the prolonged Jak2 hyperphosphorylation, in light of the modest effect that binding of SHP-1 to EpoR had on Jak2 dephosphorylation, as demonstrated in this study, and the failure of the EpoR null mutant to induce marked Jak2 hyperphosphorylation (8).

Our data from coexpressing EpoR–Jak2–SHP-1 did not allow us to distinguish whether the reduced EpoR phosphorylation was due to SHP-1 dephosphorylating the EpoR or was from a secondary effect of SHP-1 down-regulating Jak2. However, the finding that SHP-1 had no marked effect on the phosphorylation of EpoR coexpressed with c-fes suggests that the EpoR itself may not be a major SHP-1 substrate under the experimental conditions. Indeed, the phosphotyrosine sites recognized by SHP-1 SH2 domains in the cytoplasmic region of several receptors, including the EpoR, often contain hydrophobic residues upstream of the phosphotyrosine, while the PTPase catalytic domain prefers acidic amino acids at these positions (28). Thus the SH2 domains of SHP-1 may be designed to bind poor substrates of the PTPase to prevent dephosphorylation of its own docking sites.

ACKNOWLEDGMENTS

This work was supported by grants from the American Cancer Society (DB-74554) and the American Heart Association (NEO-94–074-GIA) to T. Yi and grants from the American Cancer Society Illinois Division (96-24) and the NIH (CA73381) to L.C.P.

We thank George Stark for providing Tyk2 cDNA and Christine Campbell for critical reading of the manuscript.

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