Activation of the cellular Src tyrosine kinase depends upon dephosphorylation of the carboxyl-terminal inhibitory tyrosine phosphorylation site. Herein we show that Src isolated from human platelets and Jurkat T cells is preferentially dephosphorylated at its inhibitory phosphotyrosine site by the SHP-1 tyrosine phosphatase. The data also revealed association of Src with SHP-1 in both platelets and lymphocytes and the capacity of Src to phosphorylate SHP-1 and interact with the SHP-1 NH$_2$-terminal SH2 domain in vitro. Analysis of Src activity in thymocytes from SHP-1-deficient motheaten and viable motheaten mice revealed this kinase activity to be substantially lower than that detected in wild-type thymocytes, but to be enhanced by in vitro exposure to SHP-1. Similarly, immunoblotting analysis of thymocyte Src expression before and after selective depletion of active Src protein indicated that the proportion of active relative to inactive Src protein is markedly reduced in motheaten compared with wild-type cells. These observations, together with the finding of reduced Src activity in HEY cells expressing a dominant negative form of SHP-1, provide compelling evidence that SHP-1 functions include the positive regulation of Src activation.

Both the kinase and transforming activities of the pp60$^{src}$ (Src) protein-tyrosine kinase (PTK)$^*$ are repressed by phosphorylation of a tyrosine residue within the Src carboxyl terminus (1–5). The negative regulatory effect of this phosphotyrosine (Tyr-530 and Tyr-529 in human and murine Src, respectively) has been ascribed to its association with the Src SH2 domain and consequent SH2, as well as SH3 domain-mediated intramolecular interactions that repress activity of the kinase domain (6–8). The catalytic activities of the other Src family members are similarly inhibited by phosphorylation of this conserved C-tail tyrosine (9), and activation of each of these PTKs thus depends on dephosphorylation at this site. However, while several lines of evidence implicate the Csk tyrosine kinase (10–12) as well as Src-mediated autophosphorylation (13) in the phosphorylation of the COOH-terminal regulatory tyrosine, relatively little is known about the mechanisms whereby this inhibitory phosphotyrosine residue is dephosphorylated.

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¶¶ An Arthritis Society of Canada Research Scientist. To whom correspondence should be addressed: Mount Sinai Hospital, Rm. 656A, 600 University Ave., Toronto, Ontario M5G 1X5, Canada.

†† The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; SH, Src homology domain; PACGE, polyacrylamide gel electrophoresis; PBL, peripheral blood lymphocytes; GST, glutathione $S$-transferase; Tricine, N-tris(hydroxymethyl)-methylglycine.

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chased from UBI as a recombinant protein expressed in SF9 insect cells by baculovirus containing the human c-src gene.

**Cells and Cell Lines**—The human Jurkat leukemia and the MOLT 4 T cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI 1640 (Life Technologies) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml gentamycin (Life Technologies, Inc.). Cells were grown at 37 °C in a fully humidified incubator containing 5% CO₂. Fresh human platelets and resting T cells were obtained from normal healthy peripheral blood buffy coats (Toronto Canadian Red Cross). Platelets were isolated from plasma after low speed centrifugation (PBL). T cells were isolated from peripheral blood lymphocytes (PBL) after purification by centrifugation of buffy coat blood on density gradients as described previously (33). Proliferating T cell lymphoblasts were prepared from PBL by stimulation with PHA (10 μg/ml; Difco) for 2 days followed by 100 units/ml recombinant interleukin-2 (Cetus) for an additional 2 days. The HEY protein was incubated at 37 °C for 10 min in 20 μM CNBr and subsequently isolated by SDS-PAGE.

**Immunoprecipitation**—Immunoprecipitations were performed as described previously (33). For protein detection, blots were blocked overnight in 5% nonfat milk for detection of Src, washed, incubated with the appropriate secondary antibody, and subjected to enhanced chemiluminescence (ECL) or incubation with 125I-Protein A. Where indicated, the immunoblots were stripped and reprobed with anti-Src or anti-SH-1 antibody.

**In Vivo Kinase Assay**—Tyrosine kinase activity of Src was assayed by immunoprecipitation of lysates as described above. The immunoprecipitates were then washed in kinase wash buffer (50 mM HEPES, pH 7.23, 150 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 0.5% Nonidet P-40). The precipitates were then incubated in 20 μl of kinase buffer containing 5 μCi of [γ-32P]ATP (ICN) with or without 2 μg of acid-treated rabbit muscle enolase. The mixture was incubated for 10 min at 37 °C, reduced SDS-gel sample buffer added, and the samples boiled for 10 min. The samples were centrifuged at 14,000 × g for 10 min prior to loading supernatants onto SDS-PAGE gels containing 10–12% polyacrylamide. The 32P-labeled proteins were electropheretically transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and the labeled proteins visualized by autoradiography. Src quantitation was also performed by anti-Src immunoblotting of the membranes using ECL. For assays of Src kinase activity toward SHP-1, 10 ng of recombinant Src protein was incubated at 37 °C for 10 min in 20 μl of kinase buffer containing 5 μCi of [γ-32P]ATP in the presence of acid-denatured enolase (2 μg), GST (10 μg), or GST-SHP-1 (2 μg) fusion protein. Samples were then boiled for 10 min in reduced SDS-gel sample buffer, centrifuged at 14,000 × g for 10 min, and the supernatants fractionated by SDS-PAGE. After electrophoretic transfer to Immobilon-P, Src was visualized by autoradiography.

**Western Blotting**—Western blots were performed as described in this study. Membranes were probed with 1000 units/ml polyclonal anti-p53 and 10 μg/ml monoclonal anti-pp60 c-src stained standards.

**Cyanogen Bromide (CNBr) Cleavage and Phosphoamino Acid Analysis**—In vitro [γ-32P]ATP-labeled or in vivo 32P-labeled Src was immunoprecipitated with anti-p60c-src 327 and isolated by SDS-PAGE. After electrophoretic transfer to nitrocellulose membranes, the 60-kDa Src-containing band was excised from the membranes and subjected to CNBr cleavage as described previously (34). The excised Src protein was incubated with 80 mg/ml CNBr in 70% formic acid for at least 2 h at room temperature. Samples were then washed and dried and the CNBr-generated peptide fragments resuspended in Tricine SDS sample buffer, resolved by separation on 10–20% gradient Tricine SDS-PAGE (Novex, San Diego, CA), transferred to Immobilon-P membranes, and visualized by autoradiography. For phosphoamino acid analysis, CNBr-generated protein fragments were hydrolyzed in 6 N HCl at 110 °C for 1 h and separated by one-dimensional phosphoamino acid analysis on a cellulose plate as described previously (34–36). Labeled phosphoamino acids were detected by autoradiography and identified by comparison to ninhydrin-stained standards.

**In Vivo Metabolic Labeling**—In vivo labeled Src for CNBr mapping studies was derived by incubating 4 × 10⁵ human platelets or 5 × 10⁶ Jurkat or MOLT 4 T cells for 2 h at 37 °C with 32P-OPO4 (1 mM/cell) in phosphate-free RPMI as described previously (23, 35, 36) followed by lysis in Nonidet P-40 lysing buffer and subsequent isolation of the labeled Src proteins as described above.

**GST Fusion Proteins**—The glutathione S-transferase (GST) fusion proteins were expressed in E. coli and purified by binding the following cDNA- or PCR-amplified fragments into pGEX2T: the full-length murine Src-1 cDNA (GST-SH1P-1), a full-length murine SHP-1 cDNA containing a Cys-453→Ser mutation (GST-SH1P-1 (C453S)), the SHP-1 NH-terminal SH2 domain (amino acids 1–95), the SHP-1 COOH-terminal SH2 domain (amino acids 110–205), and the SHP-1 NH- and COOH-terminal SH2 domains. The phosphatase-inactive SH2 domain was transfected into Escherichia coli JM101, and the fusion proteins purified from isopropyl-1-thio-β-p-galactopyranoside-induced bacteria with glutathione-conjugated Sepharose beads (Pharmacia Biotech Inc.). GST-SH1P-1 fusion protein was also obtained from UBI. For binding studies, 1 μg of each GST fusion protein and GST beads were incubated with 7 ng of in vitro 32P-labeled recombinant Src protein at 4 °C for 2 h. The beads were then washed and resuspended in SDS-sample buffer, and the proteins subjected to fractionation over 10% SDS-PAGE, transferred to nitrocellulose, and the 32P-labeled Src protein visualized by autoradiography.

**Phosphatase Reactions**—For phosphatase reactions using GST fusion proteins, proteins were eluted from equal amounts of GST-SH1P-1 and GST-SH1P-1 (C453S) beads into 300 μl of elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0). Equal amounts of these eluted proteins were then incubated with immunoprecipitated 32P-labeled Src (in vitro or in vivo) labeled at 37 °C in 200 μl of phosphatase buffer (10 mM Tris-Cl, 1.0 mM EDTA, 1 mg/ml bovine serum albumin, 0.1% 2-mercaptoethanol, 0.01% NaN₃, pH 7.34). To confirm the activity of the eluted proteins, activities of the PTPs were assayed in parallel using 10 mM p-nitrophenyl phosphate as substrate (37).

**Transfection Studies**—To stable transfections of HEY cells, the plasmids pCMV4Neo, pCMV4Neo-SHP-1, and pCMV4Neo-SHP-1 (C453S) were introduced by lipofection into HEY cells and the cells selected for Geneticin resistance. The resultant lines were assayed for expression of SHP-1 by immunoblotting analysis with rabbit anti-SHP-1 antibody and found to overexpress at similar levels either wild-type SHP-1 or phosphatase-inactive SHP-1 (C453S).

**RESULTS AND DISCUSSION**

To determine whether the COOH-terminal or other phosphotyrosine residues within Src are subject to dephosphorylation by SHP-1, the effects of this phosphatase on Src tyrosine phosphorylation were initially examined using CNBr cleavage analysis. As illustrated in Fig. 1A, CNBr treatment of 32P-labeled human Src has been shown previously to yield phosphorylated cleavage fragments of about 31, 9.7, and 4.7 kDa, which, respectively, contain the Src NH-terminal region encompassing the major sites for serine phosphorylation on Src, Ser-12 and Ser-17 (31-kDa fragment), the inhibitory tyrosine phosphorylation site, Tyr-530 (4.7-kDa fragment), and a key site for autophosphorylation, Tyr-419 (9.7-kDa fragment). As is consistent with these data, Tricine SDS-PAGE and subsequent phosphoamino acid analysis of the cleavage fragments derived by CNBr hydrolysis of Src immunoprecipitates from 32P-labeled cells (Molt 4 and Jurkat) and freshly-isolated platelets also revealed a predominantly phosphoserine-containing 31-kDa and predominantly phosphothreonine-containing 9.7- and 4.7-kDa fragments (Fig. 1, B and C). However, in addition to these fragments, the CNBr cleavage products generated in this analysis also included a phosphorylated fragment of about 3.3 kDa (Fig. 1B), which was shown by phosphoamino acid analysis to contain primarily phosphotyrosine (Fig. 1C). Based on the apparent molecular weight of this fragment and the locations of methionine residues within Src (Fig. 1A), the 3.3-kDa cleavage fragment most likely corresponds to amino acids 315–341 within the Src catalytic domain, a possibility that suggests Tyr-338, a residue recently shown to become phosphorylated in the context of in vitro phosphate labeling of Src (42), represents another site of Src tyrosine phosphorylation in vivo and as such...
may also be of relevance to the expression of Src kinase activity. By contrast, although one other site of tyrosine phosphorylation (corresponding to Tyr-216 in human Src) has recently been identified within the 31-kDa CNBr fragment (43), this phosphorytrosine was not detected under the conditions used in this current analysis.

As CNBr cleavage analysis under the conditions used in this study distinguished the Src region containing Tyr-530 as well as the other major known sites for tyrosine phosphorylation on Src, this approach was next used to determine whether any of these sites represent targets for SHP-1-mediated dephosphorylation in vitro. To this end, 32P-labeled recombinant Src was incubated with GST fusion proteins carrying SHP-1 or, as a control, a mutated (C453S), catalytically inert form of SHP-1, and the effects of the latter proteins on the profile of phosphorylated CNBr Src cleavage fragments then examined. As shown in Fig. 2A, the phosphorylated 9.7-, 4.7-, and 3.3-kDa CNBr fragments known to represent phosphorytrosine-containing regions of Src were again detected following CNBr hydrolysis of Src pretreated with inactive SHP-1 (C453S). By contrast, intensities of the bands representing each of these 32P-labeled species, but most notably the 4.7- and 3.3-kDa phosphorylated fragments, were markedly reduced in the context of Src pretreatment with SHP-1, a result which suggests that the phosphorytrosine residues located in these fragments can be dephosphorylated in vitro by SHP-1. Along similar lines, SHP-1 treatment of in vitro 32P-labeled Src immunoprecipitates from human platelets was also associated with dramatic decreases in the signal intensities and, by extension, phosphorylation states of the three Tyr-containing CNBr-generated cleavage fragments (Fig. 2B). As shown in Fig. 2B, phosphorylation of the Src regions represented by the 4.7- and 3.3-kDa cleavage fragments again appeared to be more sensitive to SHP-1 pretreatment than was phosphorylation of the 9.7-kDa fragment. A further assessment of these changes by densitometric analysis revealed the reduction in phosphorylation levels following a 30-min or 60-min incubation of Src with SHP-1 to be about 8–10-fold and 2-fold, respectively, greater for the 4.7- and 3.3- than for the 9.7-kDa fragment (Fig. 2C). Thus, while these data suggest the capacity of SHP-1 to dephospho-

![Figure 1](image-url)

**Fig. 1. Analysis of the phosphorylation state of Src in human platelets and T cell lines.** A, diagram of human cellular Src showing the sites for CNBr cleavage (hatched lines) and the major serine (S12 and S17) and tyrosine (Y419, Y530 and the more recently identified Y338) phosphorylation sites. The 31-, 3.3-, 9.7-, and 4.7-kDa CNBr cleavage fragments derived from Src are shown under the cleavage map, and the positions of amino acid residues flanking each fragment indicated below. B, CNBr cleavage of in vivo labeled Src. Human platelets and MOLT 4 and Jurkat T cells were metabolically labeled with 32P and, Src then isolated by immunoprecipitation. Following purification on SDS-PAGE, the labeled Src was subjected to CNBr hydrolysis and the cleavage fragments analyzed by electrophoresis over 10–20% gradient Tricine SDS-polyacrylamide, followed by transfer to Immobilon-P and autoradiography. C, phosphoamino acid analysis of the phosphorylated CNBr Src fragments indicating whether phosphorylation is on serine (S), threonine (T), or tyrosine (Y).
The SHP-1 Tyrosine Phosphatase Regulates Src Activity

2-stimulated peripheral blood lymphocytes, revealed an association of SHP-1 with Src in these cells, although comparison of the amount of SHP-1 present in total cell lysates versus the amount coprecipitated with SHP-1 suggests that the fraction of total cellular Src associated with SHP-1 is very low. SHP-1-Src association was also observed in reciprocal experiments involving anti-SHP-1 immunoblotting analysis of anti-Src immunoprecipitates (Fig. 3A, lower panel) and in similar studies of Jurkat T lymphocytes and human platelets (data not shown), but was consistently not detected when cell lysates for these studies were prepared in buffer lacking orthovanadate, a potent tyrosine phosphatase inhibitor (Fig. 3A). Together these observations link the association of SHP-1 with Src to a phosphotyrosine-dependent interaction and, as consistent with previous data showing that thrombin stimulation induces both association and increased tyrosine phosphorylation of platelet SHP-1 and Src (24), suggest that the interaction of these enzymes may be mediated through the binding of one or both of the SHP-1 SH2 domains with Src phosphotyrosine residues. To address this issue, GST fusion proteins carrying full-length SHP-1 and SHP-1 (C453S) or the tandem or single SHP-1 SH2 domains were evaluated for their capacities to interact with phosphorylated recombinant Src. The results of this in vitro analysis revealed specific binding of phosphotyrosylated Src (Fig. 3B), but not phosphorylated Fyn or Lck to the SHP-1 (C453S) fusion protein. Furthermore, as shown in Fig. 3C, Src was also precipitated by the fusion proteins containing both SH2 or the NH2-terminal SHP-1 SH2 domain, but not by the fusion protein containing only the COOH-terminal SHP-1 SH2 domain. Thus, as has also been described in relation to SHP-1 interactions with other putative substrates, such as the erythropoietin and c-Kit receptors (44, 45), these observations suggest that a single (in this instance, the NH2-terminal) SH2 domain mediates the binding of SHP-1 to phosphotyrosine(s) on Src. Conversely, GST fusion proteins containing the Src SH2 domain have been shown to precipitate phosphorylated SHP-1 from thrombin-activated platelet lysates (24), and it is therefore possible that SHP-1 association with Src involves multiple sites of interaction on these respective proteins. However, in view of our results indicating that GST-SHP-1 (C453S) fusion proteins also precipitate Src from peripheral blood lymphoblast lysates containing orthovanadate, but not from lysates lacking orthovanadate (data not shown) as well as data showing Src tyrosine activity to be substantially increased by the binding of its NH2-terminal, but not COOH-terminal SH2 domain to tyrosine-phosphorylated ligands (46), the interaction of the SHP-1 NH2-terminal SH2 domain with Src appears to represent a physiologically relevant molecular association, which may facilitate SHP-1 recruitment to and ultimately dephosphorylation of Src.

While the analysis of SHP-1 effects on Src tyrosine phosphorylation identify Src as a potential substrate for this PTP, previous data showing that SHP-1 can be tyrosine-phosphorylated by v-Src (26), the product of the transforming gene of Rous sarcoma virus, suggest that SHP-1 may also represent a substrate of the cellular Src homologue. To address this possibility, the capacity of recombinant Src to phosphorylate GST-SHP-1 fusion proteins was investigated using an in vitro kinase assay. As shown in Fig. 3D, the results of this analysis revealed the induction of SHP-1 tyrosine phosphorylation by Src, thereby identifying SHP-1 as a potential substrate for this kinase. SHP-1 has also been shown to be a substrate for Lck (47) and ZAP-70 (48), and the respective relevance of these PTKs to SHP-1 phosphorylation in vivo thus requires further analysis.

However, in view of data showing SHP-1 activity to be enhanced by tyrosine phosphorylation (49), the current data raise the possibility that a reciprocal functional relationship exists between SHP-1 and Src, the physical association of these
proteins allowing each enzyme to activate the other in either a coordinate or sequential fashion. Along similar lines, binding of SHP-1 to another putative PTK substrate (ZAP-70) has been shown to induce changes in the catalytic activities of both enzymes, although in contrast to Src, ZAP-70 kinase activity appears to be diminished in conjunction with increases in SHP-1 phosphatase activity (48).

In addition to SHP-1, a number of other PTPs, including most recently the structurally similar SHP-2 protein, have been shown to dephosphorylate Src in vitro at the COOH-terminal regulatory tyrosine (17, 18, 50, 51), but the extent to which these respective enzymes contribute to Src activation in vivo remains unclear. To specifically ascertain the biologic relevance of these respective enzymes contribution to Src activation, Molecular mass standards are indicated on the right, and the positions of Src, SHP-1, and the immunoglobulin heavy chain (IgH), are shown by arrows on the left. B, phosphorylated Src, but not Lck or Fyn, binds to SHP-1. GST fusion proteins containing the Tyr-453 → Ser catalytically inert form of SHP-1 (GST-SHP-1 (C453S)) were used to precipitate in vitro 32P-labeled recombinant Src and purified Lck and Fyn proteins. Aliquots of the phosphorylated proteins (left lane of each panel) and the GST-SHP-1 (C453S)-bound proteins (right lane of each panel) were then subjected to SDS-PAGE, transfer to Immobilon-P, and autoradiography. Use of equivalent amounts of GST-SHP-1 (C453S) was confirmed by immunoblotting with anti-GST antibody (data not shown). C, binding of phosphorylated Src to the SHP-1 SH2 domains. Glutathione-Sepharose-bound GST fusion proteins (1 μg) containing the SHP-1 NH2- and COOH-terminal (GST-SH2(N+C)) domains, only the NH2-terminal (GST-SH2(N)) or COOH-terminal (GST-SH2(C)) SH2 domains, the full-length wild-type SHP-1 (GST-SHP-1) or the catalytically inert SHP-1 (GST-SHP-1(C453S)) protein were used to precipitate in vitro 32P-labeled recombinant Src proteins (7 ng/lane) at 4 °C for 2 h; the precipitates were subjected to SDS-PAGE, transfer to Immobilon-P, and autoradiography. The positions of Src and molecular mass standards are shown on the right. D, Src phosphorylates SHP-2 in vitro. Recombinant Src (10 ng) was incubated with 2 μg of enolase, 2 μg of GST-SHP-1 fusion protein, or 10 μg of GST protein alone in kinase buffer containing 5 μCi of [γ-32P]ATP, and the samples then analyzed by SDS-PAGE and autoradiography. Arrows on the right indicate the positions of GST-SHP-1, enolase, and Src.
largely inactive. As is consistent with this contention, re-immunoprecipitation of Src from supernatants of clone 28 or SRC2 immunoprecipitates using the 327 anti-Src antibody revealed the me, but not the wild-type immunoprecipitates, to contain a substantial level of Src, presumably representing the inactive protein (Fig. 4D, panel III). These findings, together with the demonstration that the me mutation does not alter expression of other prominent thymocyte PTPs, such as CD45 and SHP-2 (Fig. 4E), provide strong evidence that SHP-1 deficiency impairs Src activation in vitro.

As SHP-1 is expressed in epithelial as well as hematopoietic lineages, the role for SHP-1 in Src activation was also investigated by evaluating Src activity in HEY ovarian cancer cells transfected with expression constructs containing cDNAs for either wild-type SHP-1 or SHP-1 (C453S), the latter of which has been shown to function in a dominant negative fashion in some cell types (48, 52). Compared with HEY cells transfected with vector alone, levels of SHP-1 protein in the cells stably transfected with SHP-1 and SHP-1 (C453S) were found to be increased by about 2-fold (data not shown). As shown in Fig. 4F, analysis of the in vitro kinase activity of Src protein immunoprecipitated from these transfectants revealed the phosphorylation of both Src as well as exogenous enolase substrate to be relatively reduced in the SHP-1 (C453S) overexpressing cells compared with cells expressing wild-type SHP-1 or transfected with vector alone. Following epidermal growth factor stimulation of these cells, Src activity again appeared lower in the SHP-1 (C453S) expressing cells than in the vector transfectants, a comparison of enolase phosphorylation with Src protein levels suggesting about 1.5–2 fold reduction in Src kinase activity in the former relative to latter cells both before and after stimulation. By contrast, phosphorylation of a number of species coprecipitated with Src from the epidermal growth factor-treated cells was markedly increased in the wild-
type SHP-1-expressing relative to empty vector-transfected cells, suggesting that Src kinase activity on endogenous substrates is enhanced in the context of SHP-1 overexpression. Together these findings strongly suggest that the effects of SHP-1 on Src tyrosine phosphorylation are relevant to the activation of SHP not only in hemopoietic, but also in epithelial cell lineages.

The data presented herein, revealing the capacity of SHP-1 to dephosphorylate Src preferentially at the COOH-terminal negative regulatory tyrosine as well as a positive correlation between the expression of SHP-1 and Src activity in vivo, provide compelling evidence that SHP-1 plays a role in the activation of Src in vivo. The identification of Src as a target for positive modulation by SHP-1, a PTP previously implicated in the negative regulation of a spectrum of signaling effectors, including the ZAP-70 and JAK2 tyrosine kinases (48, 53), indicates that SHP-1 effects on cell behavior may be realized through enhancement as well as inhibition of intracellular signaling cascades. Thus, while the structural basis for and physiologic sequelae of SHP-1-Src association require further investigation, these data suggest SHP-1 regulatory effects on Src may be relevant to the genesis of human epithelial and potentially other cancers associated with increases in Src activity.

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