SHP-1 Regulates Lck-induced Phosphatidylinositol 3-Kinase Phosphorylation and Activity*

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Ligation of the T cell antigen receptor (TCR) activates the Src family tyrosine kinase p56 Lck, which, in turn, phosphorylates a variety of intracellular substrates. The phosphatidylinositol 3-kinase (PI3K) and the tyrosine phosphatase SHP-1 are two Lck substrates that have been implicated in TCR signaling. In this study, we demonstrate that SHP-1 co-immunoprecipitates with the p85 regulatory subunit of PI3K in Jurkat T cells, and that this association is increased by ligation of the TCR complex. Co-expression of SHP-1 and PI3K with a constitutively activated form of Lck in COS7 cells demonstrated the carboxyl-terminal SH2 domain of PI3K to inducibly associate with the full-length SHP-1 protein. By contrast, a truncated SHP-1 mutant lacking the Lck phosphorylation site (Tyr365) failed to bind p85. Wild-type but not catalytically inactive SHP-1 induced dephosphorylation of p85. Furthermore, expression of SHP-1 decreased PI3K enzyme activity in anti-phosphotyrosine immunoprecipitates and phosphorylation of serine 473 in Akt, a process dependent on PI3K activity. These results indicate the presence of a functional interaction between PI3K and SHP-1 and suggest that PI3K signaling, which has been implicated in cell proliferation, apoptosis, cytoskeletal reorganization, and many other biological activities, can be regulated by SHP-1 in T lymphocytes.

In the context of appropriate co-stimulatory signals, ligation of the T cell antigen receptor (TCR) by antigenic peptide bound to a major histocompatibility complex molecule leads to T cell activation and ultimately, a functional immune response. Activation of protein tyrosine kinases and consequent intracellular protein tyrosine phosphorylation are among the first events elicited by TCR ligation and are crucial to the induction of biochemical pathways that regulate cell growth (1). This protein-tyrosine kinase activity, together with opposing protein-tyrosine phosphatase activity, plays a major role in regulating the magnitude of TCR-induced tyrosine phosphorylation, as well as the duration and termination of cell activation (1, 2). The counter-balance of tyrosine kinases by tyrosine phosphatases is integral to the maintenance of cellular homeostasis (3, 4), and disruption of this balance has been shown to be a hallmark of cellular transformation (5).

P56 Lck is a member of the Src family of non-receptor tyrosine kinases which is highly expressed in T lymphocytes (6). Along with the Fyn Src family kinase and the z-associated protein 70 (ZAP-70), Lck has been implicated in the initial activation events resulting from TCR ligation (1, 2, 6). Lck has been shown to associate with the CD4 and CD8 T cell surface antigens (6), and to play an integral role in the ligand-induced phosphorylation of the TCR intracellular components (1, 2, 6).

Indeed, Lck-mediated phosphorylation of the z subunit of the TCR and ZAP-70 couples TCR ligation to a variety of downstream signaling molecules (2), and the loss of Lck activity significantly reduces the capacity of the TCR to transduce activation signals (7).

SHP-1 is an SH2 domain-containing non-receptor tyrosine phosphatase implicated in the negative regulation of a number of growth factor receptors, including the B and T cell antigen, erythropoietin, the platelet-derived growth factor (PDGF), c-kit, and the granulocyte macrophage colony-stimulating factor receptors (8–13). SHP-1 is highly expressed in T cells (4), and has also been linked to the negative regulation of TCR signaling (14–16). This effect of SHP-1 appears to reflect its capacity to down-regulate ZAP-70 (14) and Lck (17) activities and to also dephosphorylate TCR components and downstream signaling molecules (15, 16). SHP-1 has been shown to undergo tyrosine phosphorylation in response to CD4 or CD8 stimulation as well as Lck activation (18). As is consistent with an inhibitory effect of SHP-1 on TCR signaling, thymocytes from SHP-1-deficient viable motheaten exhibit a significantly increased proliferative response to stimulation by anti-CD3 antibodies as compared with normal mouse thymocytes (16, 17).

Ligation of the TCR alters inositol lipid metabolism through induction of phosphatidylinositol 3’-kinase (PI3K) activity (1). PI3K consists of a p85 regulatory subunit with two SH2 domains and a SH3 domain, and a p110 catalytic subunit which phosphorylates the 3’-hydroxyl of the inositol ring of phosphatidylinositol (19, 20). The resulting PI3K products bind to pleckstrin homology (PH) domains of intracellular signaling molecules recruiting them to the cell membrane. Activation of the PH domain containing c-Akt (21, 22) has been associated with cell cycle progression (23, 24) and the propagation of an anti-apoptotic signal (22, 25–27). Jurkat T cell activation via anti-CD3 antibody binding to the TCR complex has been shown to result in the rapid phosphorylation of both PI3K subunits.
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(28), as well as an accumulation of PI3K products (29). TCR-induced tyrosine phosphorylation of Tyr<sup>560</sup> in the p85 subunit of PI3K and the consequent activation of PI3K have been linked to the presence of Lck (28, 30), and other recent data provide additional evidence of a role for Lck in PI3K signaling (31). However, the phosphatase(s) that dephosphorylates PI3K has not been identified as of yet.

In this study, we demonstrate that Lck activity is associated with an interaction of SHP-1 with the p85 subunit of PI3K, and also identify p85 as a target for SHP-1-mediated dephosphorylation. The association between p85 and SHP-1 requires tyrosine phosphorylation of SHP-1 and likely involves binding of SHP-1 phosphotyrosine 564 to the p85 carboxy-terminal SH2 domain via a novel tyrosine recognition motif. This interaction is also associated with a reduction in the lipid kinase activity in total anti-phosphotyrosine immunoprecipitates and a reductio

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—A monoclonal antibody against the ε chain of human CD3 complex (UCHT1, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Peter Beverley (University College, London, United Kingdom). The rabbit polyclonal antibody against Lck was described previously (32). The anti-phosphotyrosine monoclonal antibody (4G10, IgG1) and the polyclonal antibody against the p85 subunit of PI3K, and the rabbit polyclonal antibody against SHP-1 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against Akt and phospho-Akt were purchased from New England Biolabs (Beverly, MA). A monoclonal antibody against hemagglutinin (12CA5, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Bing Su (University of Texas at Houston). Rabbit anti-mouse IgG was purchased from Western Blotting Inc. (Toronto, ON). Horseradish peroxidase goat anti-mouse IgG was purchased from Bio-Rad. Glutathione-Sepharose and protein A-Sepharose beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). GST fusion proteins of HAp85 were described previously (33).

Cell Culture, Stimulation, and Lysis—

COS7 cells were transfected by Lipofection. Transient Transfection—

Antibodies and Reagents—

A monoclonal antibody against hemagglutinin (12CA5, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Bing Su (University of Texas at Houston). Rabbit anti-mouse IgG was purchased from Western Blotting Inc. (Toronto, ON). Horseradish peroxidase goat anti-mouse IgG was purchased from Bio-Rad. Glutathione-Sepharose and protein A-Sepharose beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). GST fusion proteins of HAp85 were described previously (33).

Cell Lines—Human Leukemic Jurkat T cell line E6.1, and COS7 cells were purchased from American Type Culture Collection (Rockville, MD).

Cell Culture, Stimulation, and Lysis—Jurkat T and COS7 cells were cultured in RPMI 1640 medium (Life Technologies, Inc., Grand Island, MD). Jurkat T cells were differentiated into macrophage-like cells by starvation for 24 h in the presence of PMA (20 ng/ml) and ionomycin (1 μM). Cells were then washed free of PMA and ionomycin, and stimulated with LPS (1 μg/ml) for 24 h. The supernatants were collected and used to stimulate THP-1 monocytes.

Immunoprecipitation and Immunoblotting—Detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-p85) at 4 °C for 2 h followed by another 2-h incubation with protein A-Sepharose beads. The immunoprecipitates were washed with IP wash buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Proteins were eluted from the beads by boiling in 2 × Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, MA). Membranes were blocked in 3% bovine serum albumin and incubated with anti-p85 PI3K (1:1000), anti-phosphotyrosine (1:3000), or anti-SHP-1 (1:400) at room temperature for 2 h. Horseradish peroxidase-protein A or horseradish peroxidase-antigoat IgG was used as the secondary reagent. After extensive washing, the blotted proteins were detected by enhanced chemiluminescence (ECL, Amersham). Where indicated, strips were blotted with treatment with 2% SDS and 100 mM β-mercaptoethanol in Tris-buffered saline and then rebred with anti-p85 PI3K antibodies and detected by ECL.

Fusion Protein Binding Assays—Transfected COS7 cells were starved for 24 h in serum-free medium. The cells were lysed in Nonidet P-40 lysis buffer. Bacterial lysates containing the fusion protein GST alone, the p85 amino-terminal SH2 domain, or the p85 carboxy-terminal SH2 domain were diluted in phosphate-buffered saline and incubated with glutathione-Sepharose beads. GST fusion protein beads were washed, then incubated with the transfected cell lysate at 4 °C for 2 h. After extensive washing, the proteins were eluted and immunoblotted as described above.

Immunoblotting—Cells were lysed in 1% Nonidet P-40 lysis buffer. Cell lysates normalized for protein levels (BCA assay; Pierce Chemical Co., Rockford, IL) were immunoprecipitated using anti-HA and protein A-Sepharose. Non-transfected COS7 lysate immunoprecipitates were included as a negative control. PI3K activity was determined as described (34). Briefly, the immunoprecipitates were washed sequentially in: (a) phosphate-buffered saline, 100 μM Na<sub>2</sub>VO<sub>4</sub>, 1% Triton X-100; (b) 100 mM Tris, pH 7.6, 0.5 mM L<sub>Cl</sub>C, 100 μM Na<sub>3</sub>VO<sub>4</sub>; (c) 100 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 100 μM Na<sub>2</sub>VO<sub>4</sub>; (d) 20 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 30 mM Na<sub>3</sub>PO<sub>4</sub>, 200 μM Na<sub>3</sub>VO<sub>4</sub> 1 mM phenylmethylsulfonyl fluoride, 0.03% Triton X-100, and resuspended in 30 μl of kinase reaction buffer (33 μl Tris, pH 7.6, 125 mM NaCl, 15 μM Mg<sub>2</sub>Cl<sub>2</sub>, 200 μM adenosine, 15 μM ATP, 30 μCi of [γ<sup>32</sup>P]<ATP>; (ATP). Phosphatidylinositol (PI) was resuspended in 20 μl of Hepes, pH 7.5, at 2 mg/ml and sonicated on ice for 10 min. The PI 3-kinase reaction was initiated by adding 10 μl of the PI suspension. The reaction proceeded for 30 min at room temperature and was terminated by adding 100 μl of 1 N HCl. Lipids were extracted by 600 μl of chloroform:methanol (1:1). The organic phase was washed with H<sub>2</sub>O, collected and dried by vacuum centrifugation. The lipids were resuspended in 20 μl of chloroform: methanol: acetone: water (60:47:2:11.3). Radiolabeled phosphatidylinositol phosphate was visualized by autoradiography.

Lck Autophosphorylation Assay—Cells were lysed in kinase lysis buffer (35). Cell lysates normalized for protein levels were immunoprecipitated using a rabbit antibody against human Lck and protein A-Sepharose. Non-transfected COS7 and SHP-1 transfected cell lysates were also subjected to immunoprecipitation, the beads were washed four times with wash buffer including 1% Nonidet P-40, 150 mM NaCl, 50 mM Hepes, pH 7.5, 1 mM Na<sub>2</sub>VO<sub>4</sub>). The washed beads were then resuspended in 50 μl of kinase reaction mixture (20 μl Hepes, pH 7.4, 100 mM NaCl, 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 5 μM ATP, 10 μCi of [γ<sup>32</sup>P]<ATP>) and incubated at room temperature for 30 min. The reaction was stopped by washing the beads twice with wash buffer including 1 mM EDTA. Proteins were eluted from the beads by boiling in 2 × Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA). Radiolabeled Lck was visualized by autoradiography.

Subcellular Fractionation—Jurkat cells were incubated in serum-free RPMI for 16 h prior to stimulation. Cells were divided into two aliquots (25 × 10<sup>6</sup> cell each), and one was stimulated by cross-linking TCR complex proteins with anti-CD3 (see above) for 7 min. Membrane and cytosolic fractions were separated based on the protocol of Resh and Erickson (36). Briefly, cells were washed twice with STE (150 mM NaCl, 50 mM Tris, 1 mM EDTA) and collected with low speed centrifugation (1,000 × g). The cells were resuspended in hypotonic lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM KC1, 1 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.4) and incubated on ice for 15 min. The cells were lysis with a French homogenizer. Lysates were adjusted to 0.25 μM sucrose, 1 mM EDTA, and centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was removed, and the pellet resuspended in 0.25 μM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, and given five additional strokes in a Dounce homogenizer, and centrifuged at 1,000 × g for 10 min at 4 °C. The supernatants were combined and centrifuged at 100,000 × g for 1 h. The result-
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RESULTS

SHP-1 Physically Associates with PI3K—Although PI3K has been shown to be phosphorylated and activated following TCR ligation (28), the phosphatase responsible for dephosphorylation of PI3K has yet to be identified. The tyrosine phosphatase SHP-1 has been shown to target a number of molecules required for TCR signal relay (4). To address the possibility that SHP-1 associates with PI3K in TCR-stimulated Jurkat cells, we investigated by cross-linking the TCR complex with antibodies to CD3. We utilized a subcellular fractionation approach (36) to maximize the yield of activated, membrane-associated PI3K in Jurkat cells (19, 20, 37, 38), either directly or as part of a multimeric complex. Whether the baseline association of these proteins reflects constitutive activation of Jurkat cells, even in serum-free medium, remains to be determined.

The p85 Carboxyl-terminal SH2 Domain Binds Phosphorylated SHP-1—To determine the functional relationship between PI3K and SHP-1, we used a transient transfection system involving the expression of recombinant p85 and SHP-1 in COS7 cells. T cell receptor activation was simulated in this system by overexpression of a constitutively activated form of Lck (Lck Y505F) that was generated by mutating the regulatory carboxyl-terminal inhibitory tyrosine (6). In previous studies, the regulatory PI3K subunit p85 has been shown to be phosphorylated by Lck Y505F when these proteins are co-expressed in COS1 cells (30). The major site of Lck-induced p85 phosphorylation has been mapped to a tyrosine residue (Tyr^{688}) located within the carboxyl-terminal SH2 domain (30). As Tyr^{664} in the p85 carboxyl-terminal tail is also phosphorylated by Lck, and both p85 and SHP-1 contain SH2 domains, Lck-induced physical association of p85 with SHP-1 might be mediated by binding of the p85 SH2 domain(s) to phosphotyrosine on SHP-1. Alternatively, the SH2 domain of SHP-1 might inducibly associate with phosphorylated p85. To distinguish between these possibilities, the capacity of GST fusion proteins containing the p85 amino- or carboxyl-terminal SH2 domains to precipitate SHP-1 from lysates of transfected COS7 cells was examined. For these studies, the cells were transfected with a catalytically inactive form of SHP-1 (SHP-1 C453S) so as to prevent autodephosphorylation (18) and thus maximize the level of SHP-1 phosphorylation. As illustrated by the anti-SHP-1 Western blot shown in Fig. 2A, the results of this analysis revealed only the carboxyl-terminal SH2 domain of p85 to bind SHP-1 C453S, and indicated this association to require the presence of Lck Y505F. By contrast, tyrosine-phosphorylated p85 was not precipitated by GST-SHP-1 SH2 domain fusion proteins (data not shown). To determine whether the major site on SHP-1 for Lck-mediated phosphorylation (18) was involved in the p85 SH2-mediated association between p85 and SHP-1, a truncation mutant construct (SHP-1 ∆35) encoding amino acids 1 through Lys^{560} of SHP-1 and thus lacking Tyr^{664} was derived and its capacity to associate with the p85 carboxyl-terminal SH2 domain then examined in the transfected COS7 cells. As illustrated by Fig. 2B, immunoblot analysis revealed the failure of SHP-1 ∆35 to associate with the p85 carboxyl-terminal domain, and thus demonstrated this association to require one or more amino acids mapping within the ∆35 segment. As Tyr^{664}, located within the last 35 amino acids of SHP-1, is the primary site of Lck phosphorylation in SHP-1, and Lck is required for the association of SHP-1 with the carboxyl-terminal SH2 domain of PI3K (Fig. 2A), these data strongly suggest that it is the interaction of this phosphorylated residue with the p85 carboxyl-terminal SH2 domain which mediates physical association of p85 with SHP-1.

SHP-1 dephosphorylates Lck-phosphorylated p85—Association of the p85 SH2 domain with the carboxyl terminus of SHP-1 creates the opportunity for SHP-1 to dephosphorylate Tyr^{688} of p85 (Fig. 2C), the major site of Lck phosphorylation on p85 (30). Accordingly, the possibility that SHP-1 dephosphorylates Lck-phosphorylated p85 was investigated in COS7 cells co-transfected with a recombinant hemagglutinin epitope tagged p85 construct (Hap85), Lck Y505F, and SHP-1. Immunoprecipitation of Hap85, followed by SDS-PAGE and Western blotting with anti-phosphotyrosine clearly demonstrate the co-
transfection of HAp85 with Y505F to induce a level of tyrosine phosphorylation of the recombinant p85 protein which is significantly increased relative to the vector control (Fig. 3A, lanes 1 and 2). Expression of SHP-1 with Y505F and HAp85 in this system was associated with a reduction of p85 phosphorylation to a level comparable to that detected in vector control cells (Fig. 3A, lanes 1 and 3). Thus p85 appears to represent a SHP-1 substrate. Interestingly, substitution of wild-type SHP-1 with SHP-1 C453S not only restored p85 phosphorylation to the level detected in the Y505F/HAp85 lysate, but also engendered the highest p85 phosphorylation detected in any transfectant (Fig. 3A).

As p85 heterodimerizes with the p110 subunit of PI3K, the possibility that association with p110 was required for SHP-1-mediated dephosphorylation of p85 was also studied. To this end, the Lck Y505F transfected COS7 cells were also co-transfected with a mutant form of p85 (ΔHAp85) (39) in which the inter-SH2 (iSH2) p110-binding region, that is absolutely required for p85 heterodimerization (Fig. 2C), was deleted. Analysis of these cells revealed ΔHAp85 to be both phosphorylated by activated Lck, and dephosphorylated by SHP-1 (Fig. 3B). Thus, while the physical association between p110 and SHP-1 cannot be excluded, these data suggest that such an association is not necessary for the SHP-1-mediated dephosphorylation of p85.

Although the Lck Y505F mutant used in these studies lacks the regulatory carboxyl tyrosine, it is possible that the effects of SHP-1 on p85 phosphorylation relate to SHP-1-mediated dephosphorylation of other phosphotyrosine sites in Lck and consequent down-regulation of Lck Y505F activity. To assess this possibility, Y505F autophosphorylation in vitro was examined in COS7 cells transfected with Lck Y505F alone or in combination with either SHP-1 or SHP-1 C453S. The results of this assay revealed the in vitro kinase activity of Lck Y505F to remain intact in the presence of SHP-1 expression (Fig. 3C). Taken together, these data indicate that p85 not only physically associates with SHP-1, but also is dephosphorylated by SHP-1.

Effect of SHP-1 Expression on PI3K Activity—To determine whether SHP-1-mediated dephosphorylation of p85 is associated with a change in PI3K activity, epitope-tagged p85 was immunoprecipitated from COS7 co-transfectants and the kinase activity of the associated p110 catalytic subunit was evaluated using an in vitro lipid phosphorylation assay. The results of this analysis revealed PI3K lipid kinase activity to be unaffected by SHP-1 expression (data not shown). However, as SHP-1 interaction with PI3K involves PI3K tyrosine phosphorylation, the possibility that SHP-1 binding diminishes activity of phosphorylated, but not total cellular PI3K, was also addressed. To this end, anti-phosphotyrosine antibodies were used to immunoprecipitate phosphorylated proteins from the COS7 lysates, and the precipitated phosphoproteins were then...
evaluated for lipid kinase activity. Results of this analysis revealed the lipid kinase activity present in the tyrosine-phosphorylated fraction to be markedly reduced in the Lck Y505F/SHP-1 co-transfectants as compared with the transfectants in which Lck Y505F was expressed in the absence of SHP-1 (Fig. 4A). By contrast, expression of SHP-1 C453S did not affect anti-phosphotyrosine immunoprecipitable lipid kinase activity, a result which indicates the decreased PI3K activity observed in the Lck Y505F/SHP-1 cells to be dependent on the phosphatase activity of SHP-1.

The regulatory effects of SHP-1 on PI3K signaling were also investigated by analyzing the relevance of SHP-1 to the activities of signaling molecules downstream of PI3K. Most notable among the latter proteins is Akt, a PIK domain-containing kinase linked to cell cycle progression, proliferation, and cell death (40). Phosphorylation of Akt at serine residue 473 (S473) is absolutely dependent on PI3K activity (22), being abrogated by PI3K inhibitors LY294002 and wortmannin (data not shown). Evaluation of PI3K-dependent Akt Ser^{473} phosphorylation thus provides a surrogate assay for PI3K activity in intact cells. To explore the effects of SHP-1 on PI3K-induced Akt phosphorylation, hemagglutinin-tagged Akt (HA-Akt) and Lck Y505F were co-transfected in COS7 cells and the phosphorylation of Akt examined by immunoblotting analysis using an anti-Akt antibody specifically recognizing phosphoserine 473. Results of this analysis (Fig. 4B) revealed Lck Y505F co-transfection to be associated with a modest increase in Akt Ser^{473} phosphorylation. By contrast, co-expression of wild-type SHP-1 with Lck Y505F and HA-Akt reduced phospho-Akt to a level similar to that detected in cells transfected with HA-Akt alone. Interestingly, expression of SHP-1 C453S in conjunction with Lck Y505F and HA-Akt was associated with increases in levels of phospho-Akt exceeding those detected in cells expressing Lck Y505F and HA-Akt (Fig. 4, B and C). These latter findings parallel the observations revealing Lck Y505F effects on p85 phosphorylation (Fig. 3A) to be somewhat enhanced in the context of SHP-1 C453S expression, a finding which suggests that substrate trapping by the latter protein may impact on PI3K signaling.

**DISCUSSION**

In the current study, the possibility that interaction between PI3K and SHP-1 contributes to the effects of these respective proteins on TCR signaling was investigated. The data reveal that SHP-1 interacts with the p85 subunit of PI3K in Jurkat T cells, and indicate this association to be enhanced by TCR stimulation. Furthermore, SHP-1 and PI3K are present in a complex including the TCR. Association of SHP-1 with PI3K was also found to be inducible in COS7 cells by addition of activated Lck and to represent a phosphotyrosine-dependent interaction involving association of the p85 carboxyl-terminal SH2 domain likely with phosphorylated tyrosine 564 in the SHP-1 carboxy-terminal tail. By further analysis of this interaction in COS7 cells, p85 was identified as a substrate for SHP-1, and the activity of tyrosine-phosphorylated PI3K shown to be markedly reduced in the presence of wild-type, but not catalytically inert SHP-1 (41). SHP-1 expression did not, however, alter lipid kinase activity of total cellular PI3K. A role for SHP-1 in regulating PI3K signaling was also evidenced by the finding that SHP-1 expression in COS7 cells engenders a decrease in phosphorylation of Akt Ser^{473}. Phosphorylation of Akt at this site involves association of the Akt PH domain with phosphorylated PI3K lipid substrates in the cell membrane and is known to be completely dependent on PI3K activation (22). Taken together, these observations provide evidence that SHP-1 not only interacts with PI3K, but also impacts upon PI3K activation and downstream signaling.

The current data indicate the SHP-1/PI3K interaction to be mediated by binding of the PI3K p85 subunit carboxyl-terminal SH2 domain to phosphorylated SHP-1 and to require that the most carboxyl-terminal located 35-amino acid segment of SHP-1 be intact. As Tyr^{564} which has been identified as the primary target for Lek effects on SHP-1, maps within this region (18), it appears likely that Tyr^{564} represents the site on SHP-1 which interacts with the p85 SH2 domain. Interestingly, the results of these studies also revealed the truncated SHP-1 Δ35 protein to exhibit decreased phosphatase activity (data not shown), a result which contrasts with previous data.
suggesting catalytic activity of this mutant form of SHP-1 to be enhanced (42). This discrepancy may reflect the differences in the conditions used for the respective phosphatase assays, the previous study involving analysis of PTP activity at pH 5.5. In the current study, the assay was performed at pH 7.3, which would presumably more closely approximate physiologic conditions. In any case, in view of the potential for this truncation mutation to alter SHP-1 activity, the SHP-1 ΔΔ5 protein was used here only in binding studies, and its effects on p85 phosphorylation and PI3K activity were not examined.

Although p85 SH2 domains have been previously shown to specifically target YMXM phosphotyrosine motifs, the current study data suggest that the carboxyl-terminal SH2 domain of p85 binds a SHP-1 phosphotyrosine residue (Tyr564) embedded within a YENV motif. This divergence in the SH2 domain specificity is, however, not without precedent (30, 43). The SHP-1 SH2 domains, for example, have been demonstrated to interact with several distinct phosphotyrosine motifs (44). Furthermore, in vitro phosphorylation of the p85 carboxyl-terminal SH2 domain has been shown to alter its capacity to bind certain targets in activated Jurkat cells (30), a finding which again raises the possibility that the SH2 domain may interact with phosphotyrosines in more than one structural context.

Interestingly, p85 association with SHP-1 in PDGFR-stimulated MCP-7 cells has been shown to be mediated by binding of the SHP-1 amino-terminal SH2 domain to phosphorylated p85 (10). By contrast, interaction of the SHP-1 SH2 domains with phosphorylated p85 was not detected in the current study, a discrepancy which may reflect differences in the PI3K sites targeted by Lck and PDGFR, respectively (18, 45). It is also not clear whether p85 is a direct PDGFR target in vivo. However, taken together, these findings raise the possibility that association of SHP-1 with PI3K and the consequent modulation of PI3K signaling occurs in a variety of cell stimulatory contexts.

The data reported here concur with other data in the literature revealing the phosphorylation of p85 and the in vitro lipid kinase activity of immunoprecipitated PI3K to be poorly correlated (30). However, wild-type SHP-1 decreases PI3K activity in anti-phosphotyrosine immunoprecipitates and PI3K-dependent phosphorylation of Akt in intact cells. Interestingly, both p85 phosphorylation and PI3K activity, as revealed by Akt S473 phosphorylation, were found to be up-regulated in the presence of catalytically inactive SHP-1 C453S protein. As SHP-1 C453S does not enhance activity of Lck Y505F (Fig. 3C), these data suggest that SHP-1 C453S acts in this context as a “substrate trap,” binding phosphorylated targets, but failing to dephosphorylate or release these phosphoproteins, thus protecting them from dephosphorylation by other cellular phosphatases. The increased level of phospho-Akt in the SHP-1 C453S-transfected cells may also reflect the capacity of mutant SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of PI3K bound SHP-1 C453S to induce conformational changes in PI3K which favor its activation, possibly by mimicking the effects of a positive modulator of PI3K. Both of these latter hypotheses suggest the involvement of a third molecule in the PI3K/SHP-1 interaction, a possibility also suggested by our finding that SHP-1 and PI3K can be co-immunoprecipitated from the membrane fraction of resting, serum-starved Jurkat cells in which protein phosphorylation would be expected to be minimal. Therefore, SHP-1 may also associate with PI3K by a phosphotyrosine-independent mechanism, such as interactions with an SH3 domain containing protein (46). This possibility however, remains purely speculative at present.

In summary, the data shown here reveal a functional relationship between Lck, SHP-1, and PI3K signaling proteins, which have each been identified as key elements in the induction of T cell activation. While Lck acts primarily to promote TCR signaling (6), SHP-1 effects on TCR signal relay are largely inhibitory (16, 17). The current data suggest that this inhibitory effect of SHP-1 is realized at least in part through the down-regulation of PI3K activity. However, in view of the limited understanding of the role for PI3K activity in TCR signaling, further studies are required to address the physiological significance of SHP-1 effects on PI3K. It also remains to be determined whether SHP-1 effects on PI3K signaling in vivo reflect direct modulation of PI3K activity by SHP-1 and/or the capacity of SHP-1 to influence other PI3K modulatory signals, or, alternatively, the consequence of PI3K association with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator.
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1071–1076
35. Cone, J. C., Lu, Y., Trecilayan, J. M., Bjorndahl, J. M., and Phillips, C. A. (1993) Eur. J. Immunol. 23, 2488–2497
36. Resh, M. D., and Erikson, R. L. (1985) J. Cell Biol. 100, 409–417
37. Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. A., and Williams, L. T. (1996) Mol. Cell. Biol. 16, 4117–4127
38. Carpenter, C. L., and Cantley, L. C. (1996) Biochim. Biophys. Acta 1288, M11–M16
39. Klippel, A., Escobedo, J. A., Hu, Q., and Williams, L. T. (1993) Mol. Cell. Biol. 13, 5560–5566
40. Hemmings, B. A. (1997) Science 275, 628–630
41. Pani, G., Kozlowski, M., Cambier, J. C., Mills, G. B., and Siminovitch, K. A. (1995) J. Exp. Med. 181, 2077–2084
42. Pei, D., Lorenz, U., Klingmuller, U., Neel, B. G., and Walsh, C. T. (1994) Biochemistry 33, 15483–15493
43. Rameh, L. E., Chen, C. S., and Cantley, L. C. (1995) Cell 83, 821–830
44. Kozlowski, M., Larose, L., Lee, F., Le, D. M., Rottapel, R., and Siminovitch, K. A. (1998) Mol. Cell. Biol. 18, 2088–2099
45. Bouchard, P., Zhao, Z., Banville, D., Dumas, F., Fischer, E. H., and Shen, S. H. (1994) J. Biol. Chem. 269, 19585–19589
46. Fusaki, N., Iwamatsu, A., Iwashima, M., and Fujisawa, J. (1997) J. Biol. Chem. 272, 6214–6219