Negative Regulation of PYK2/Related Adhesion Focal Tyrosine Kinase Signal Transduction by Hematopoietic Tyrosine Phosphatase SHPTP1

Shailendra Kumar, Shalom Avraham‡, Ajit Bharti, Jaya Goyal§, Pramod Pandey, and Surender Kharbanda¶

From the Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, the Division of Experimental Medicine and Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts 02115, and the Department of Radiation Oncology, New England Medical Center, Boston, Massachusetts 02111

Related adhesion focal tyrosine kinase (RAFTK) (also known as PYK2) is a cytoplasmic tyrosine kinase related to the focal adhesion kinase (FAK) p125Src. RAFTK is rapidly phosphorylated on tyrosine residues in response to various stimuli, such as tumor necrosis factor-α, changes in osmolarity, elevation in intracellular calcium concentration, lysophosphatidic acid, and bradykinin. Overexpression of RAFTK induces activation of c-Jun amino-terminal kinase (also known as stress-activated protein kinase), mitogen-activated protein kinase (MAPK), and p38 MAPK. The present studies demonstrate that RAFTK binds constitutively to the protein tyrosine phosphatase SHPTP1. In contrast to PTP1B, overexpression of wild-type SHPTP1 blocks tyrosine phosphorylation of RAFTK. The results further demonstrate that RAFTK is a direct substrate of SHPTP1 in vitro. Moreover, treatment of PC12 cells with bradykinin is associated with inhibition in tyrosine phosphorylation of RAFTK in the presence of SHPTP1. Furthermore, in contrast to the phosphatase-dead SHPTP1 C453S mutant, overexpression of wild-type SHPTP1 blocks interaction of RAFTK with the SH2-domain of c-Src and inhibits RAFTK-mediated MAPK activation. Significantly, cotransfection of RAFTK with SHPTP1 did not inhibit RAFTK-mediated c-Jun amino-terminal kinase activation. Taken together, these findings suggest that SHPTP1 plays a negative role in PYK2/RAFTK signaling by dephosphorylating RAFTK.

Protein tyrosine kinases are critical components of signaling pathways that control cellular proliferation, differentiation, and apoptosis. Related adhesion focal tyrosine kinase (RAFTK), also known as proline-rich tyrosine kinase (PYK2), calcium-dependent tyrosine kinase, and cellular adhesion kinase β is a recently described cytoplasmic tyrosine kinase that is homologous to the focal adhesion kinase (FAK) (1–4). RAFTK, 116-kDa kinase, is selectively expressed in hematopoietic cells and neurons and is distinct from FAK (2, 5). RAFTK lacks a transmembrane domain and, similar to FAK, does not have any SH2 or SH3 domains (2). FAK and RAFTK share 45% overall sequence identity and 60% identity in the catalytic domain. Certain key tyrosine residues are conserved between RAFTK and FAK that function as c-Src and Grb2 SH2 domain binding sites (1, 2, 4, 6–13). Moreover, studies have shown that, in rat hippocampal slices and cortical synaptosomes, RAFTK and FAK are regulated differentially by pathways involving calcium and protein kinase C (14). Recent studies have described identification of an another isoform of Pyk2, Pyk2-H, that is implicated in chemokine and antigen receptor signaling (15).

RAFTK has been shown to display integrin-dependent phosphorylation in B lymphocytes, CMK cells, and transfected COS cells (16, 17). In contrast, RAFTK phosphorylation has been found to be independent of integrin ligation during platelet aggregation (18). Other studies have demonstrated that RAFTK is tyrosine-phosphorylated following β2 integrin or B cell antigen receptor-mediated stimulation in both transformed and normal B cells (16, 19). More recent work has shown that RAFTK is activated by intracellular calcium, treatment with tumor necrosis factor-α, ultraviolet light, or hyperosmolality (1, 11, 20). RAFTK is involved in calcium-stimulated regulation of ion channels (1). RAFTK also regulates stress-induced JNK and p38 MAPK activation in PC12 cells (11, 20–22).

Tyrosine phosphorylation of RAFTK by lysophosphatidic acid or bradykinin stimulation contributes to activation of the MAPK pathway (11). Furthermore, tyrosine phosphorylation of RAFTK by lysophosphatidic acid, bradykinin, or fluoroualuminate (23) leads to binding of the SH2 domain of Src to Tyr-402 (autophosphorylation site (1) of RAFTK and activation of Src (11). Since overexpression of RAFTK Y402F mutant failed to activate MAPK, these findings, taken together, indicated that RAFTK tyrosine phosphorylation and RAFTK-mediated MAPK activation depend on induction of c-Src kinase activity by binding of c-Src to autophosphorylated Tyr-402 on RAFTK. However, the molecular mechanisms that regulate the tyrosine phosphorylation of Tyr-402 of RAFTK are presently unclear.

The present studies have addressed the involvement of a

tathione S-transferase; PIRS, preimmune rabbit serum; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; HI, heat-inactivated; HA, hemagglutinin.

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tyrosine phosphatase, SHPTP1, in RAFTK-mediated signaling. The results demonstrate that RAFTK binds constitutively to SHPTP1.

We also show that, by contrast to PTP1B, overexpression of SHPTP1 blocks tyrosine phosphorylation of RAFTK and its subsequent interaction with the SH2 domain of Src. Furthermore, SHPTP1 selectively inhibits certain functions of RAFTK, such as RAFTK-mediated activation of MAPK but not of JNK.

MATERIALS AND METHODS

Cell Culture—Human U-937 myeloid leukemia cells were grown in RPMI 1640 supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. PC12 cells were grown in RPMI 1640 containing 10% HI horse serum, 5% HI-FBS, and antibiotics. 293T or PC12 cells (1 × 106/100-mm culture dish) were plated 24 h before treating with bradykinin (Sigma). Cells (1 × 106/100 mm culture dish) were plated 24 h before transfection with various cDNAs. U-937 or PC12 cells were treated with 1 μg bradykinin for 5 min.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitations were performed as described (24). In brief, cells were washed with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/ml of leupeptin and aprotinin) as described. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK (2), anti-SHPTP1 (Upstate Biotechnology, Inc., Lake Placid, NY), or preimmune rabbit serum (PIRS), and the resultant protein precipitates were analyzed by immunoblotting with anti-SHPTP1 or anti-RAFTK antibodies. To determine the stoichiometry of interactions, lysates were also immunoprecipitated with anti-RAFTK or anti-SHPTP1, and lysates before and after immunoprecipitation were analyzed by immunoblotting with anti-SHPTP1 or anti-RAFTK antibodies, respectively.

Transient Transfections—293T cells were grown in 100-mm cell culture dishes, and cells were seeded a day before transfections. Cells were transiently transfected with FLAG-SHPTP1 and FLAG-RAFTK by calcium phosphate as described (25). After 12 h of incubation at 37 °C, the medium was replaced, and the cells were incubated for another 24–36 h. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK, anti-SHPTP1, or PIRS, and the precipitates were analyzed by immunoblotting with anti-FLAG antibody. 293T cells were transiently transfected with wild-type FLAG-RAFTK, kinase-dead FLAG-RAFTK (RAFTK Y402F), or an autophosphorylation dead mutant of RAFTK (RAFTK Y402F) with or without wild-type FLAG-SHPTP1 (SHPTP1) or phosphatase-dead mutant of FLAG-SHPTP1 (SHPTP1 C-S). Cells were also separately transfected with HA-PTP1B (26) and FLAG-RAFTK as a negative control. Lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-Tyr(P) antibody. Anti-FLAG immunoprecipitates were also analyzed by immunoblotting with anti-RAFTK antibody. Anti-FLAG immunoprecipitates were also analyzed by immunoblotting with anti-RAFTK. PC12 cells were transiently transfected with wild-type FLAG-RAFTK, kinase-dead FLAG-RAFTK (RAFTK Y402F) with or without wild-type FLAG-SHPTP1 (SHPTP1) or phosphatase-dead mutant of FLAG-SHPTP1 (SHPTP1 C-S). Cells were also separately transfected with HA-PTP1B (26) and FLAG-RAFTK as a negative control. Lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-Tyr(P). The autoradiograms were scanned, and the percentage inhibition in bradykinin-induced tyrosine phosphorylation of RAFTK was expressed as the mean ± S.D of three independent experiments.

Dephosphorylation of RAFTK in Vitro—293T cells were grown in 100-mm cell culture dishes, and cells were seeded a day before transfections. Cells were transiently transfected with FLAG-RAFTK by calcium phosphate. Cell lysates were prepared in Nonidet P-40 lysis buffer without phosphatase inhibitors. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK antibody, and precipitated proteins were sedimented with protein A-Sepharose beads. The resultant immune complexes were incubated in an assay buffer containing [γ-32P]ATP for 30 min at room temperature. Following the autophosphorylation reactions, the beads were extensively washed to remove excess ATP, and immunopurified autophosphorylated RAFTK was then incubated with buffer or column purified SHPTP1 for another 30 min. The reactions were terminated by the addition of SDS sample buffer and analyzed by 7.5% SDS-PAGE and autoradiography. Anti-RAFTK immunoprecipitates were also incubated with purified SHPTP1 in the presence of cold ATP, and the reaction products were analyzed by immunoblotting with anti-Tyr(P) antibody.

RAFTK Activity Assays—RAFTK Activity Assays were performed as described (24). In brief, cells were washed with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/ml of leupeptin and aprotinin) as described. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK (2), anti-SHPTP1 (Upstate Biotechnology), and the resultant immunoprecipitates were then analyzed by immunoblotting with anti-RAFTK. B, U-937 cell lysates were immunoprecipitated with PIRS, anti-RAFTK, or anti-SHPTP1, and the resulting immunoprecipitates were then analyzed by immunoblotting with anti-RAFTK or anti-SHPTP1. Lysates from U-937 cell lysates were subjected to immunoprecipitation with anti-RAFTK, and the resultant protein precipitates were analyzed by immunoblotting with anti-RAFTK antibody. D, 293T cells were transiently transfected with wild-type FLAG-RAFTK, kinase-dead FLAG-RAFTK (RAFTK Y402F), or an autophosphorylation dead mutant of RAFTK (RAFTK Y402F) with or without wild-type FLAG-SHPTP1 (SHPTP1) or phosphatase-dead mutant of FLAG-SHPTP1 (SHPTP1 C-S). Cells were also separately transfected with HA-PTP1B (26) and FLAG-RAFTK as a negative control. Lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-Tyr(P) antibody. The autoradiograms were scanned, and the percentage inhibition in bradykinin-induced tyrosine phosphorylation of RAFTK was expressed as the mean ± S.D of three independent experiments.

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(41) Sigma (1) as a substrate for 15 min at 30 °C. The reactions were terminated by the addition of SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography.

c-Jun Kinase Assays—293T cells were transiently transfected with various amounts of RAFTK and pEBG-SAPK with or without SHPTP1 or SHPTP1 C-S. After 48 h, total cell lysates were prepared and incubated with 5 μg of immobilized GST for 30 min at 4 °C. The protein complexes were washed with lysis buffer and incubated in kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂) containing [γ-32P]ATP for 15 min at 30 °C using GST-Jun-(1–102) as substrate as described. Kinase reactions were terminated by addition of SDS-PAGE sample buffer, and phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

MAPK Assays—293T cells were transiently transfected with various amounts of FLAG-RAFTK and HA-MAPK with or without wild-type SHPTP1 or SHPTP1 C453S mutant. Total cell lysates were then subjected to immunoprecipitation with anti-HA antibodies for 2 h at 4 °C. The protein complexes were then incubated in kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂) containing [γ-32P]ATP for 15 min at 30 °C using myelin basic protein (MBP) as substrate. The protein complexes were washed with lysis buffer and incubated in kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂) containing [γ-32P]ATP for 15 min at 30 °C. The protein complexes were then incubated in kinase buffer containing [γ-32P]ATP and MBP as substrate. After 15 min, the protein complexes were then subjected to SDS-PAGE and analyzed by autoradiography. Total lysates were also separately analyzed by immunoblotting with anti-Raf, anti-HA, or anti-SHPTP1 antibodies.

c-Src/RAFTK Binding Assays in Vivo—PC12 cells were transiently cotransfected with FLAG-RAFTK and wild-type SHPTP1 or SHPTP1 C453S mutant. Following transfections, cells were treated with 1 μM bradykinin for 5 min. Total cell lysates were subjected to immunoprecipitation with anti-c-Src antibody and analyzed by immunoblotting with anti-RAFTK antibody. The protein bands were scanned by densitometer, and signal intensities were plotted and expressed as arbitrary units ± S.D from three independent experiments.

GST-Src SH2 Domain Fusion Protein Binding Assays—Lysates from transiently transfected 293T cells were incubated with affinity purified GST-c-Src-SH2 domain (Upstate Biotechnology) fusion protein linked to glutathione-Sepharose beads as described. The resulting protein complexes were washed three times with lysis buffer containing 0.1% detergent and boiled for 5 min in SDS sample buffer. The complexes were then separated by SDS-PAGE and subjected to immunoblot analysis with anti-Tyr(P) or anti-RAFTK antibodies.

Src SH2 Domain Binding Assays in Vitro—293T cells were transiently transfected with FLAG-RAFTK. Total cell lysates were prepared and subjected to immunoprecipitation with anti-RAFTK. The resulting immune complexes were incubated in an assay buffer containing [γ-32P]ATP for 30 min at room temperature. Following the autophosphorylation reactions, the beads were extensively washed to remove excess ATP and then incubated with buffer or column purified SHPTP1 for 30 min. Following dephosphorylation reactions, RAFTK protein was eluted from the protein A-Sepharose beads by 0.5% SDS elution buffer. Reaction mixtures were then diluted with lysis buffer to a final concentration of 0.1% SDS and then incubated with GST-Src-SH2 domain fusion protein for 45 min. Following extensive washing, the beads were boiled in SDS sample buffer and analyzed by 7.5% SDS-PAGE and autoradiography.

RESULTS AND DISCUSSION

U-937 cell lysates were subjected to immunoprecipitation with anti-RAFTK antibody and analyzed by immunoblotting
with anti-SHPTP1. The results demonstrated reactivity with a 70-kDa protein (Fig. 1A). In the reciprocal experiment, analysis of anti-SHPTP1 immunoprecipitates with anti-RAFTK confirmed constitutive interaction between these two proteins (Fig. 1B). Since treatment with bradykinin induces activation of RAFTK (1), we investigated whether bradykinin affects the interaction between RAFTK and SHPTP1. The results demonstrate that treatment of PC12 cells with bradykinin is associated with little, if any, increase in the association of RAFTK with SHPTP1 (Fig. 1C). The association between RAFTK and SHPTP1 was further analyzed by overexpression of wild-type FLAG-SHPTP1 and FLAG-RAFTK in human embryonic kidney 293T cells that do not express RAFTK (20). Whole cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-FLAG antibody. Incubation of total cell lysates with preimmune rabbit serum was used as a negative control. Anti-SHPTP1 immunoprecipitates and total cell lysate were used as positive controls. Reactivity at 70 kDa with anti-FLAG immunoblot in anti-RAFTK immunoprecipitates confirmed association of these two proteins (Fig. 1D).

To evaluate the stoichiometry of the interaction between RAFTK and SHPTP1, we subjected U-937 cell lysates to immunoprecipitation with anti-RAFTK, and we analyzed the supernatants before and after immunoprecipitation by immunoblotting with anti-SHPTP1. Signal intensities from lysates before and after anti-RAFTK immunoprecipitation were compared by laser densitometric scanning. The results demonstrated that approximately 60% (average of three independent experiments) of RAFTK is associated with SHPTP1 (Fig. 2A). Similar results were obtained in a reciprocal experiment in which the cell lysates were subjected to immunoprecipitation with anti-SHPTP1, and we analyzed the supernatants before and after immunoprecipitation by immunoblotting with anti-RAFTK (Fig. 2B).

The increase in tyrosine phosphorylation of RAFTK in response to various inducers has suggested that RAFTK is activated by phosphorylation on tyrosine (1, 3, 20, 13). As demonstrated previously, overexpression of RAFTK in 293T cells leads to approximately a 12–15-fold induction of its tyrosine phosphorylation (Fig. 3A). RAFTK autophosphorylates itself on Tyr-402 (11). As expected, overexpression of RAFTK Y402F or dominant negative RAFTK K-M mutants in 293T cells did not increase their level of tyrosine phosphorylation (Fig. 3A). The ability of SHPTP1 to bind to RAFTK suggests that this protein might be a physiological target for SHPTP1. To address this issue, we cotransfected 293T cells with wild-type RAFTK and SHPTP1 or phosphatase-dead SHPTP1 (SHPTP1 C453S) (31) and analyzed anti-RAFTK immunoprecipitates by immunoblotting with anti-phosphotyrosine. Importantly, in contrast to SHPTP1 C453S mutant, the level of tyrosine-phosphorylated RAFTK is substantially reduced in 293T cells transfected with wild-type SHPTP1 (Fig. 3B, top panel). The blots were then stripped and reprobed with anti-RAFTK antibodies to ensure that the immunoprecipitates contained equal levels of RAFTK (Fig. 3B, middle panel). Total cell lysates were also analyzed by immunoblotting with anti-SHPTP1 (Fig. 3B, bottom panel). Since overexpression of SHPTP1 can nonspecifically dephosphorylate all potential targets within the cell, 293T cells were transfected with RAFTK with or without SHPTP1, and total cell lysates were analyzed by immunoblotting with anti-Tyr(P). As a control, RAFTK Y402F mutant was separately transfected

Fig. 5. SHPTP1 regulates tyrosine phosphorylation of RAFTK in vitro. A, PC12 cells were treated with 1 μM bradykinin (Brad.) and harvested at the indicated times. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK, and the immunoprecipitates were analyzed by immunoblotting (IB) with anti-Tyr(P). B, PC12 cells were treated with 1 μM bradykinin and harvested at the indicated times. Total cell lysates were subjected to immunoprecipitation with anti-MAPK, and immune complex kinase assays were performed using MBP as substrate. C, PC12 cells were transfected with wild-type (wt) or C453S mutant (C-S) of SHPTP1 and then treated with 1 μM bradykinin for 5 min. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK, and the precipitates were analyzed by immunoblotting with anti-SHPTP1. The results demonstrated reactivity with a 70-kDa protein (Fig. 1). Since treatment with bradykinin induces activation of RAFTK (1), we investigated whether bradykinin affects the interaction between RAFTK and SHPTP1. The results demonstrate that treatment of PC12 cells with bradykinin is associated with little, if any, increase in the association of RAFTK with SHPTP1 (Fig. 1C). The association between RAFTK and SHPTP1 was further analyzed by overexpression of wild-type FLAG-SHPTP1 and FLAG-RAFTK in human embryonic kidney 293T cells that do not express RAFTK (20). Whole cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-FLAG antibody. Incubation of total cell lysates with preimmune rabbit serum was used as a negative control. Anti-SHPTP1 immunoprecipitates and total cell lysate were used as positive controls. Reactivity at 70 kDa with anti-FLAG immunoblot in anti-RAFTK immunoprecipitates confirmed association of these two proteins (Fig. 1D).

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The increase in tyrosine phosphorylation of RAFTK in response to various inducers has suggested that RAFTK is activated by phosphorylation on tyrosine (1, 3, 20, 23). As demonstrated previously, overexpression of RAFTK in 293T cells leads to approximately a 12–15-fold induction of its tyrosine phosphorylation (Fig. 3A). RAFTK autophosphorylates itself on Tyr-402 (11). As expected, overexpression of RAFTK Y402F or dominant negative RAFTK K-M mutants in 293T cells did not increase their level of tyrosine phosphorylation (Fig. 3A). The ability of SHPTP1 to bind to RAFTK suggests that this protein might be a physiological target for SHPTP1. To address this issue, we cotransfected 293T cells with wild-type RAFTK and SHPTP1 or phosphatase-dead SHPTP1 (SHPTP1 C453S) (31) and analyzed anti-RAFTK immunoprecipitates by immunoblotting with anti-phosphotyrosine. Importantly, in contrast to SHPTP1 C453S mutant, the level of tyrosine-phosphorylated RAFTK is substantially reduced in 293T cells transfected with wild-type SHPTP1 (Fig. 3B, top panel). The blots were then stripped and reprobed with anti-RAFTK antibodies to ensure that the immunoprecipitates contained equal levels of RAFTK (Fig. 3B, middle panel). Total cell lysates were also analyzed by immunoblotting with anti-SHPTP1 (Fig. 3B, bottom panel). Since overexpression of SHPTP1 can nonspecifically dephosphorylate all potential targets within the cell, 293T cells were transfected with RAFTK with or without SHPTP1, and total cell lysates were analyzed by immunoblotting with anti-Tyr(P). As a control, RAFTK Y402F mutant was separately transfected
in 293T cells. The results demonstrate that many other phosphotyrosyl proteins are not affected by SHPTP1 expression, indicating that this phosphatase does not indiscriminately dephosphorylate all potential targets within the cell (Fig. 3C, upper panel). This effect was without any difference in the RAFTK protein levels (Fig. 3C, lower panel). Moreover, to confirm specificity of SHPTP1, a non-SH2 domain containing tyrosine phosphatase, PTP1B, was overexpressed with RAFTK, and anti-RAFTK immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P). The results demonstrate that, by contrast to SHPTP1, overexpression of PTP1B failed to block tyrosine phosphorylation of RAFTK (Fig. 3D). Taken together, these findings indicated that activation of RAFTK by autophosphorylation on Tyr-402 is specifically regulated by SHPTP1 in vitro.

Whereas the present findings demonstrate that overexpression of wild-type SHPTP1 is associated with inhibition of autophosphorylation of RAFTK, we asked whether RAFTK is a direct target of SHPTP1 in vitro. To address this issue, we immunopurified RAFTK from 293T cells transiently overexpressing RAFTK and incubated with purified recombinant phosphatase-active SHPTP1 protein in the presence of cold or [$\gamma^{32}$P]ATP. RAFTK autophosphorylation was determined in these reactions either by immunoblotting with anti-Tyr(P) or by SDS-PAGE and autoradiography, respectively. The results demonstrate that autophosphorylation of RAFTK is inhibited in the presence of SHPTP1 in vitro (Fig. 4A and B). Since purified SHPTP1 dephosphorylates RAFTK autophosphorylation site Tyr-402 in vitro, we asked whether dephosphorylation of RAFTK affects its kinase function. To address this, we first incubated purified SHPTP1 with immunopurified RAFTK in the presence of ATP. After washing, RAFTK activity was measured in a kinase reaction containing [$\gamma^{32}$P]ATP and poly(Glu-Tyr) (4:1) as a substrate (1). The results demonstrate little, if any, change in RAFTK-mediated phosphorylation of poly(Glu-Tyr) in the presence or absence of SHPTP1 (Fig. 4C). Taken together, these findings indicate the following: (i) RAFTK is a direct substrate of SHPTP1, and (ii) dephosphorylation of RAFTK by SHPTP1 does not inhibit its kinase activity.

To assess the functional significance of the interaction of RAFTK with SHPTP1 in vivo, we asked whether SHPTP1 regulates tyrosine phosphorylation of RAFTK induced in response to bradykinin. To address this issue, PC12 cells were transfected with wild-type or C453S mutant of SHPTP1 and then treated with bradykinin. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-Tyr(P). Three independent experiments were performed, and signal intensities were determined by densitometric analysis. The results demonstrate that treatment of PC12 cells with bradykinin is associated with induction of tyrosine phosphorylation of RAFTK (Fig. 5A) as well as induction of MAPK activity (Fig. 5B). Importantly, by contrast to SHPTP1 C453S mutant, analysis of anti-RAFTK immunoprecipitates with anti-Tyr(P) in cells overexpressing wild-type SHPTP1 demonstrated over 3-fold inhibition in tyrosine phosphorylation of RAFTK (Fig. 5C, left and right panels). Collectively, these findings support SHPTP1-mediated dephosphorylation of RAFTK in vitro as well as in response to bradykinin.

Few insights are available regarding the functional role of RAFTK. Recent studies have shown that RAFTK regulates stress-induced c-Jun amino-terminal protein kinase (JNK) activation (11, 20). As demonstrated previously, the finding that overexpression of RAFTK in 293T cells induces phosphorylation of GST-Jun (20) supported RAFTK-mediated activation of JNK in these cells (Fig. 6A). The activation of JNK was dependent on expression of the RAFTK protein (Fig. 6A, upper panel). Increased activation of JNK in these assays was without any change in JNK protein levels (Fig. 6A, lower panel). Since RAFTK is activated by certain agents such as tumor necrosis factor-α or UV light and contributes to activation of JNK (11, 20–22), we asked whether SHPTP1 is involved in regulation of this stress pathway. To address this issue, we cotransfected pEG-SAPK and RAFTK with SHPTP1 or SHPTP1 C453S mutant and assayed glutathione-Sepharose
that regulate RAFTK-mediated MAPK activation. Previous studies have shown that c-Raf-1 acts upstream of the MAPK pathway (32–34). Therefore, it is possible that the inhibitory effect of SHPTP1 on RAFTK-mediated activation of MAPK, demonstrated in the present study, could be a result of SHPTP1-mediated dephosphorylation of other targets downstream of RAFTK, such as c-Raf-1. To address this issue, 293T cells were transiently transfected with HA-MAPK with c-Raf-1 and wild-type or C453S mutant of SHPTP1. Following transfections, total cell lysates were subjected to immunoprecipitation with anti-HA, and in vitro immune complex kinase assays were performed using MBP as a substrate. The results demonstrate that overexpression of wild-type SHPTP1 has little, if any, effect on c-Raf-1-mediated activation of MAPK (Fig. 7C). Taken together, these findings suggested that RAFTK-dependent activation of MAPK is inhibited by SHPTP1-mediated dephosphorylation of RAFTK.

Previous studies have demonstrated that RAFTK tyrosine phosphorylation and RAFTK-mediated MAPK activation depend on c-Src kinase activity stimulated by binding to auto-phosphorylated Tyr-402 on RAFTK (11). Therefore, we next examined the role of SHPTP1 in regulating the interaction of RAFTK with c-Src. To address this issue, RAFTK protein was immunopurified by transiently overexpressing FLAG-RAFTK in 293T cells. Immunopurified RAFTK was incubated in a kinase buffer containing [γ-32P]ATP at 30 °C for 30 min. Following extensive washing, autophosphorylated RAFTK protein was incubated with purified SHPTP1 protein for an additional 30 min. The resulting dephosphorylated RAFTK protein was eluted from the beads and subjected to incubation with GST-c-Src-SH2 domain fusion protein. Following fusion protein-binding reactions, the bound proteins were analyzed by autoradiography. The results demonstrate that incubation of SHPTP1 with RAFTK significantly inhibits the interaction of c-Src-SH2 domain with RAFTK (Fig. 8A). In vitro binding experiments using a GST fusion protein containing the SH2 domain of c-Src were also performed in lysates overexpressing wild-type RAFTK with or without wild-type or C453S SHPTP1. Lysate from overexpressing RAFTK Y402F mutant was separately used as control. GST fusion protein containing the SH2 domain of c-Src was associated with binding to tyrosine-phosphorylated RAFTK but not to the RAFTK Y402F mutant (Fig. 8B). In contrast to SHPTP1 C453S mutant, cotransfection of RAFTK with wild-type SHPTP1 completely blocked RAFTK interaction with the SH2 domain of c-Src (Fig. 8B). To address further this issue in vivo, PC12 cells were transiently transfected with FLAG-RAFTK and wild-type SHPTP1 or SHPTP1 C453S mutant. Cells were treated with bradykinin, and total cell lysates were subjected to immunoprecipitation with anti-c-Src antibody and analyzed by immunoblotting with anti-FLAG. The protein bands were scanned by densitometer, and signal intensities were plotted and expressed as arbitrary values ± S.D from three independent experiments. D, 293T cells were transfected with SHPTP1 and wild-type or Y402F mutant of RAFTK. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-SHPTP1.
that SHPTP1 inhibits recruitment of c-Src to RAFTK by blocking the interaction of the SH2-domain of c-Src and not by competing for binding to RAFTK.

Our results suggest that the tyrosine phosphatase SHPTP1 regulates RAFTK-mediated apoptosis. In this context, recent studies have shown that overexpression of a tyrosine kinase Csk (negative regulator of Src; see Ref. 35) inhibits RAFTK-mediated MAPK activation and further indicate the role of Src in mediating RAFTK response. The present results indicate that SHPTP1 plays an important role in RAFTK-mediated MAPK activation. Our findings also demonstrate that SHPTP1-mediated dephosphorylation of RAFTK on Tyr-402 is not associated with inhibition of the kinase function of RAFTK. Other studies have shown that kinase activity of RAFTK is necessary for RAFTK-mediated induction of JNK (11). In concert with these findings, the present results indicate that RAFTK binds competitively to SHPTP1 and tyrosine kinase activity of RAFTK is required for RAFTK-mediated MAPK activation. Our findings also demonstrate that SHPTP1-mediated dephosphorylation of RAFTK on Tyr-402 is not associated with inhibition of the kinase function of RAFTK. Other studies have shown that kinase activity of RAFTK is necessary for RAFTK-mediated induction of JNK.

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