

# Negative Regulation of the SHPTP1 Protein Tyrosine Phosphatase by Protein Kinase C $\delta$ in Response to DNA Damage

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## ABSTRACT

The SHPTP1 protein tyrosine phosphatase is activated by the c-Abl and Lyn tyrosine kinases in the cellular response to genotoxic stress. However, signaling mechanisms involved in the negative regulation of SHPTP1 are unknown. This study demonstrates that protein kinase C  $\delta$  (PKC $\delta$ ) associates with SHPTP1. The PKC $\delta$  catalytic domain binds directly to SHPTP1. The results also demonstrate that PKC $\delta$  is required, at least in part, for phosphorylation and inactivation of SHPTP1. The phosphatase activity of SHPTP1 was attenuated by coinubation with PKC $\delta$  in vitro. In addition, treatment of U-937 human

myeloid leukemia cells with 1- $\beta$ -D-arabinofuranosylcytosine (ara-C) was associated with induction of the PKC $\delta$  kinase function and inhibition of SHPTP1 activity. Down-regulation of SHPTP1 by ara-C was blocked by the PKC $\delta$  inhibitor rottlerin but not by the PKC $\alpha$  and - $\beta$  inhibitor Gö6976. Moreover, transient coexpression studies with a dominant-negative mutant of PKC $\delta$  demonstrate that the kinase activity of PKC $\delta$  is required for the down-regulation of SHPTP1. These findings support the functional interaction between PKC $\delta$  and SHPTP1 in the cellular response to DNA damage.

Protein tyrosine phosphatases are a highly diverse family of enzymes that play essential roles in the regulation of cell proliferation, differentiation, and transformation (Frearson and Alexander, 1997; Neel, 1997). SHPTP1 (Plutzky et al., 1992), also referred to as PTP1C (Shen et al., 1991), HCP (Yi et al., 1992), or SHP (Matthews et al., 1992), is a member of a subfamily of protein tyrosine phosphatases that contains two Src homology 2 domains at the N terminus (Fischer et al., 1991). SHPTP1 is predominantly expressed in hematopoietic cells (Matthews et al., 1992; Plutzky et al., 1992; Yi et al., 1992) and may play a crucial role in hematopoiesis (Shultz et al., 1993; Tsui et al., 1993). Certain insights are available regarding protein tyrosine kinases that regulate SHPTP1. Previous studies have shown that SHPTP1 is regulated by various tyrosine kinases, including Src (Somani et al., 1997), Lck (Lorenz et al., 1994), Lyn (Yoshida et al., 1999), Syk (Dustin et al., 1999), Zap-70 (Plas et al., 1996), c-Abl (Kharbanda et al., 1996), Tyk2 (David et al., 1995), Jak1, and Jak2 (Klingmuller et al., 1995). Findings showing that the Lyn and c-Abl tyrosine kinases phosphorylate and activate SHPTP1 in cells treated with DNA-damaging agents

support a role for SHPTP1 as a downstream signal in the stress response (Kharbanda et al., 1996; Yoshida et al., 1999). Recent studies have also demonstrated that the induction of SHPTP1 activity by genotoxic stress negatively regulates the activation of Lyn, c-Abl, and the stress-activated protein kinase pathway (Kharbanda et al., 1996; Liedtke et al., 1998; Yoshida et al., 1999). These findings indicate that SHPTP1 functions as a negative regulator in the response to DNA damage.

Treatment of human tumor cells with DNA-damaging agents is associated with the induction of apoptosis (Kaufmann, 1989; Gunji et al., 1991). Efforts to define the role of protein kinase C (PKC) have been complicated by the expression of multiple isoforms in different cells and their involvement in both pro- and antiapoptotic signaling cascades. PKC $\delta$  is a type of novel PKC that is activated by diacylglycerol or 12-*O*-tetradecanoylphorbol 13-acetate but is calcium-independent (Newton, 1995). Recent studies have demonstrated that PKC $\delta$  is cleaved in the third variable region by caspase 3 in the cellular response to DNA damage (Emoto et al., 1995, 1996). The cleaved catalytic fragment of PKC $\delta$  is constitutively active and induces apoptosis in various cell lines (Ghayur et al., 1996). These findings support a role for

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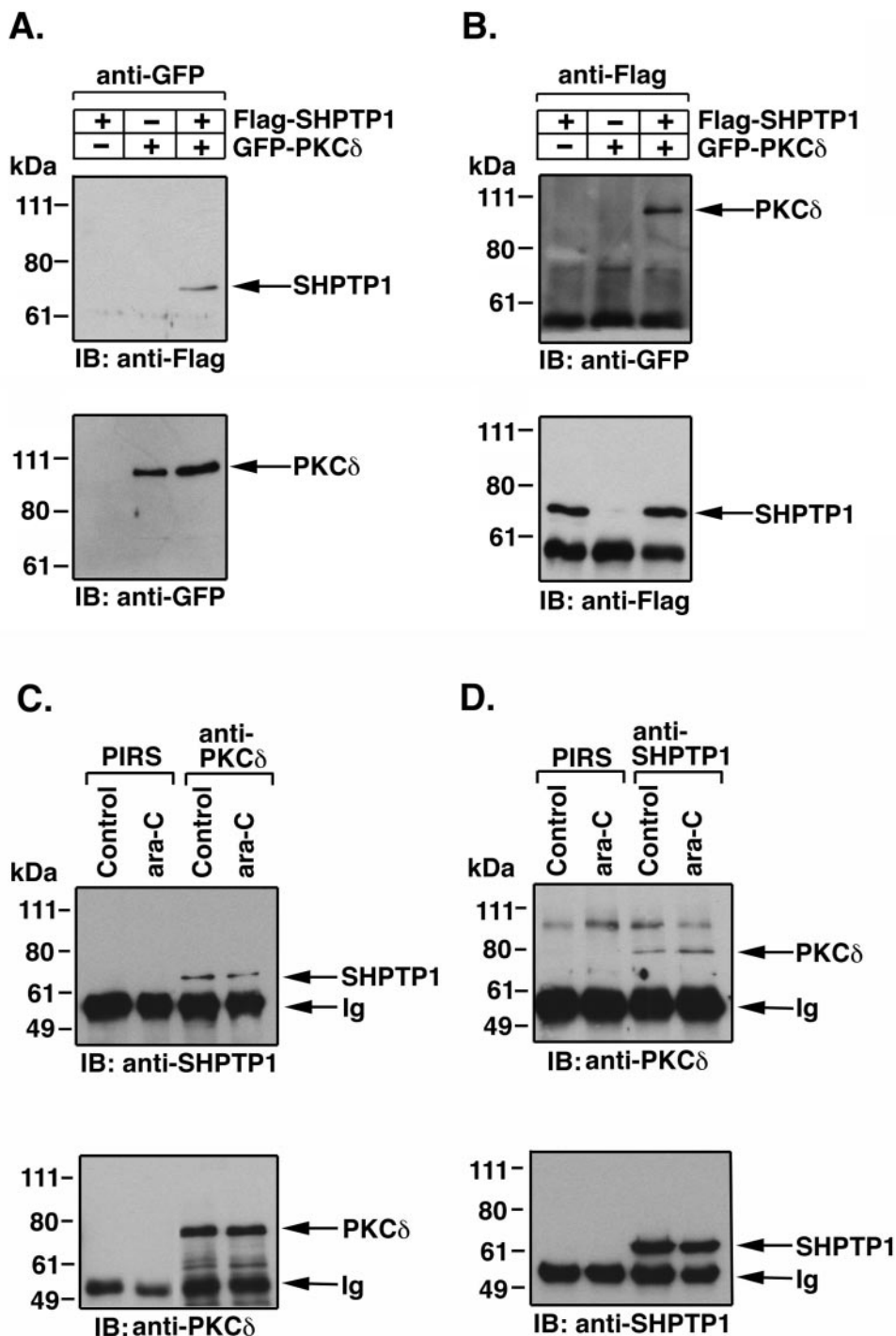
**ABBREVIATIONS:** PKC $\delta$ , protein kinase C  $\delta$ ; ara-C, 1- $\beta$ -D-arabinofuranosylcytosine; PKC, protein kinase C; CF, catalytic fragment; PBS, phosphate-buffered saline; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid; GST, glutathione S-transferase; FL, full-length; RD, regulatory domain; Flag-SHPTP1, Flag-tagged SHPTP1; GFP, green fluorescent protein; GFP-PKC $\delta$ , green fluorescent protein-tagged PKC $\delta$ .

PKC $\delta$  in the apoptotic response to genotoxic stress (Emoto et al., 1995, 1996; Ghayur et al., 1996).

This study demonstrates that PKC $\delta$  interacts with SHPTP1. The results show that the C-terminal catalytic fragment of PKC $\delta$  is responsible for direct binding to SHPTP1. We also show that phosphorylation of SHPTP1 by PKC $\delta$  is associated with the down-regulation of SHPTP1 tyrosine phosphatase activity. The results also demonstrate that activation of PKC $\delta$  by genotoxic stress is required, at least in part, for the associated down-regulation of SHPTP1.

## Experimental Procedures

**Cell Culture.** Human U-937, U-937/neo, and U-937/Bcl-x<sub>L</sub> (Datta et al., 1995) myeloid leukemia cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine. Embryonal 293T kidney cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Cells were treated with 10  $\mu$ M ara-C (Sigma-Aldrich, St. Louis, MO), 10  $\mu$ M rottlerin (Calbiochem, San Diego, CA), or 50 nM Gö6976 (Calbiochem).



**Fig. 1.** Association of SHPTP1 with PKC $\delta$ . A and B, 293T cells were transfected with Flag-SHPTP1 and/or GFP-PKC $\delta$ . Cell lysates were immunoprecipitated with anti-GFP (A) or anti-Flag (B). Immune complexes were subjected to immunoblotting (IB) with anti-Flag and anti-GFP. C and D, U-937 cells were treated with 10  $\mu$ M ara-C and harvested at 1 h. Lysates from control and treated cells were immunoprecipitated with preimmune rabbit serum (PIRS), anti-PKC $\delta$  (C), or anti-SHPTP1 (D). The precipitates were subjected to immunoblotting with anti-SHPTP1 and anti-PKC $\delta$ .

**Cell Transfections.** 293T cells were transiently cotransfected with pcDNA3-Flag-SHPTP1 (Yoshida et al., 1999), pGFP (CLONTECH, Palo Alto, CA), pGFP-PKC $\delta$  catalytic fragment (CF), and/or pGFP-PKC $\delta$  CF(K-R) (Bharti et al., 1998) using the calcium phosphate method. PKC $\delta$  CF(K-R) is kinase-inactive mutant in which the lysine residue at position 378 in the putative ATP-binding site has been substituted with arginine by site-directed mutagenesis (Bharti et al., 1998). At 36 h after transfection, cells were left untreated or were treated with ara-C for the indicated times and then harvested for preparing cell lysates.

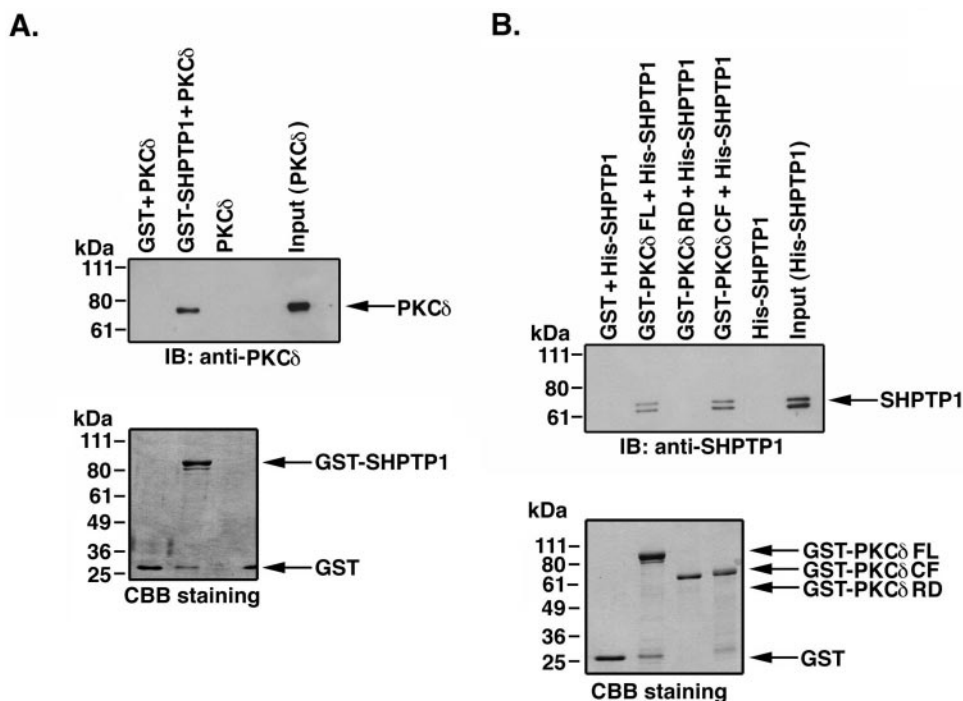
**Immunoprecipitation and Immunoblot Analysis.** Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed on ice for 30 min in lysis buffer [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 0.05% sodium deoxycholate, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM  $\beta$ -glycerophosphate, and 10  $\mu$ g/ml each of aprotinin, leupeptin, and pepstatin A]. The lysates were cleared by centrifugation at 14,000 rpm for 15 min. Soluble proteins were incubated with anti-PKC $\delta$  (sc-937; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-SHPTP1 (sc287; Santa Cruz Biotechnology), anti-Flag (Sigma-Aldrich), or anti-GFP (Roche Molecular Biochemicals, Somerville, NJ) antibodies for 2 to 6 h at 4°C followed by 1 h of incubation with protein A/G-Sepharose beads (Santa Cruz Biotechnology). The immune complexes were washed three times with the lysis buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose filters. The residual binding sites were blocked by incubating with 5% dry milk in PBS including 0.05% Tween 20 overnight with gentle shaking at 4°C. The filters were incubated with the antibodies listed above for 1 to 4 h at room temperature. After washing three times with the PBS and 0.05% Tween 20 solution, the filters were incubated with anti-rabbit or anti-mouse IgG peroxidase conjugate (Santa Cruz Biotechnology). The antigen-antibody complexes were visualized by using chemiluminescence (PerkinElmer Life Science Products, Boston, MA).

**In Vitro Binding Assays.** Glutathione *S*-transferase (GST) and GST-SHPTP1 (2  $\mu$ g; Upstate Biotechnology, Lake Placid, NY) were incubated with full-length recombinant PKC $\delta$  (Calbiochem) in lysis buffer for 1 h at 4°C. The adsorbed material obtained by washing three times with lysis buffer was separated by SDS-PAGE and an-

alyzed by using immunoblotting with anti-PKC $\delta$  antibody. In reciprocal experiments, GST, GST-PKC $\delta$  full-length (FL), GST-PKC $\delta$  regulatory domain (RD), and GST-PKC $\delta$  CF (Bharti et al., 1998) (2  $\mu$ g each) were incubated with His-SHPTP1 (Kharbanda et al., 1996). The complexes were washed and then separated by SDS-PAGE and subjected to immunoblot analysis with anti-SHPTP1 antibody. Input of the GST fusion proteins was monitored by the use of SDS-PAGE and Coomassie brilliant blue staining.

**In Vitro Kinase Assays.** Biologically active full-length recombinant PKC $\delta$  was prepared from insect Sf-9 cells infected with a baculovirus containing the PKC $\delta$  cDNA (Calbiochem). The recombinant PKC $\delta$  preparation contains phospholipids and has a specific activity of >800 units/mg of protein (according to the manufacturer). One unit of PKC $\delta$  activity is defined as the amount of enzyme that transfers 1.0 nmol of phosphate to the PKC $\delta$  peptide substrate per minute at 37°C. GST-SHPTP1 (5  $\mu$ g) or His-SHPTP1 (2  $\mu$ g) proteins were incubated in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 2 mM DTT, and 0.1 mM sodium vanadate) with GST-PKC $\delta$  CF or recombinant PKC $\delta$  and [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; PerkinElmer Life Science Products) for 15 min at 30°C. Alternatively, PKC $\delta$  kinase assays were performed in the absence of other proteins to assess autophosphorylation or in the presence of histone H1 (Calbiochem) as a substrate. Where indicated, PKC $\delta$  kinase assays were also performed in the presence of PKC activators (10  $\mu$ M phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidyl serine, and Triton X-100 mixed micelles; Invitrogen, Carlsbad, CA). For the analysis of PKC $\delta$  activity in the anti-PKC $\delta$  immunoprecipitates, the immune complexes were washed three times with lysis buffer and once with kinase buffer and resuspended in kinase buffer containing 2 to 5 Ci of [ $\gamma$ -<sup>32</sup>P]ATP and His-SHPTP1 or histone H1. The reaction mixtures were incubated for 30 min at 30°C and terminated by the addition of SDS sample buffer. Reaction products were separated by the use of SDS-PAGE and analyzed by the use of autoradiography.

**SHPTP1 Phosphatase Assays.** In vitro SHPTP1 tyrosine phosphatase assays were performed using the Malachite Green Phosphatase Assay (Upstate Biotechnology) with phosphopeptide (RRLIE-DAEpYAARG) as a substrate. For in vivo phosphatase assays, cells were disrupted in lysis buffer. The lysates were incubated with anti-SHPTP1 or anti-Flag antibodies for 2 h at 4°C followed by 1 h of



**Fig. 2.** PKC $\delta$  binds directly to SHPTP1. A, glutathione beads containing GST or GST-SHPTP1 were incubated with recombinant PKC $\delta$ . The adsorbates were analyzed by immunoblotting with anti-PKC $\delta$ . B, glutathione beads containing GST, GST-PKC $\delta$  FL, GST-PKC $\delta$  RD, or GST-PKC $\delta$  CF were incubated with column-purified His-SHPTP1. The adsorbates were analyzed by immunoblotting with anti-SHPTP1. Input of the GST fusion proteins was monitored by SDS-PAGE and Coomassie brilliant blue (CBB) staining (bottom).

incubation with protein A/G-Sepharose beads. The immune complexes were washed three times with lysis buffer without phosphatase inhibitor and once with phosphatase buffer [40 mM MES, pH 5.0, 1.6 mM DTT] and resuspended in phosphatase buffer containing the phosphopeptide. The reaction mixtures were incubated for 30 min at room temperature and were terminated by the addition of Malachite Green solution. Absorbance was measured in a spectrophotometer at 620 nm. Phosphate release was determined by comparing absorbance with that obtained with the phosphate standard.

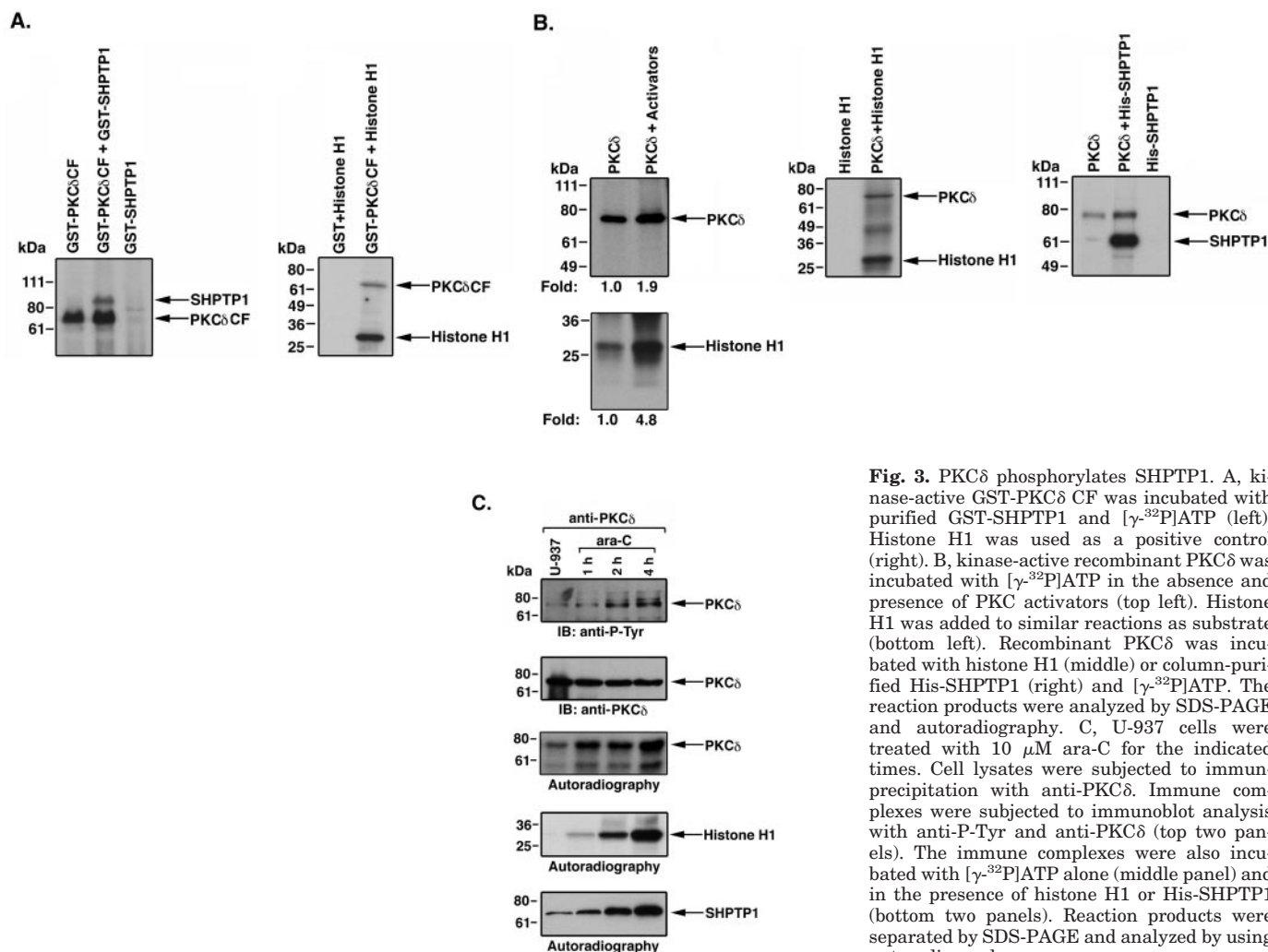
## Results

**SHPTP1 Interacts Directly with PKC $\delta$ .** To assess potential interactions between SHPTP1 and PKC $\delta$  in cells, lysates from 293T cells cotransfected with Flag-tagged SHPTP1 (Flag-SHPTP1) and GFP-tagged PKC $\delta$  (GFP-PKC $\delta$ ) were subjected to immunoprecipitation with anti-GFP antibody. Immunoblot analysis of the complexes with anti-Flag demonstrated the detection of SHPTP1 (Fig. 1A). The reciprocal experiment, in which anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-GFP confirmed coimmunoprecipitation of SHPTP1 and PKC $\delta$  (Fig. 1B). To determine whether endogenous SHPTP1 associates with endogenous PKC $\delta$ , anti-PKC $\delta$  immunoprecipitates from human U-937 cells were subjected to immunoblotting with anti-SHPTP1. The results demonstrate that PKC $\delta$  associates with

SHPTP1 from both control and ara-C-treated cells (Fig. 1C). Immunoblot analysis of anti-SHPTP1 immunoprecipitates with anti-PKC $\delta$  provided further support for constitutive binding of endogenous SHPTP1 and PKC $\delta$  (Fig. 1D). These findings demonstrate that SHPTP1 binds constitutively to PKC $\delta$  in cells.

To investigate whether the association of SHPTP1 and PKC $\delta$  is direct, glutathione beads containing GST or GST-SHPTP1 were incubated with recombinant PKC $\delta$ . An analysis of the adsorbates by immunoblotting with anti-PKC $\delta$  demonstrated a direct interaction between SHPTP1 and PKC $\delta$  (Fig. 2A). To define the region of PKC $\delta$  responsible for binding to SHPTP1, glutathione beads containing GST-PKC $\delta$  FL, GST-PKC $\delta$  RD, or GST-PKC $\delta$  CF were incubated with His-SHPTP1. An analysis of the adsorbates with anti-SHPTP1 demonstrated binding to PKC $\delta$  FL and PKC $\delta$  CF, but not to PKC $\delta$  RD (Fig. 2B). These findings indicate that SHPTP1 interacts directly with the C-terminal PKC $\delta$  catalytic region.

**PKC $\delta$  Phosphorylates SHPTP1.** To determine whether SHPTP1 is phosphorylated by PKC $\delta$  in vitro, kinase-active GST-PKC $\delta$  CF was incubated with GST-SHPTP1 and [ $\gamma$ - $^{32}$ P]ATP. GST-PKC $\delta$  CF lacks the regulatory domain and is constitutively active in the absence of cofactors (Emoto et al., 1995). Analysis of the reaction products by SDS-PAGE



**Fig. 3.** PKC $\delta$  phosphorylates SHPTP1. A, kinase-active GST-PKC $\delta$  CF was incubated with purified GST-SHPTP1 and [ $\gamma$ - $^{32}$ P]ATP (left). Histone H1 was used as a positive control (right). B, kinase-active recombinant PKC $\delta$  was incubated with [ $\gamma$ - $^{32}$ P]ATP in the absence and presence of PKC activators (top left). Histone H1 was added to similar reactions as substrate (bottom left). Recombinant PKC $\delta$  was incubated with histone H1 (middle) or column-purified His-SHPTP1 (right) and [ $\gamma$ - $^{32}$ P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. C, U-937 cells were treated with 10  $\mu$ M ara-C for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-PKC $\delta$ . Immune complexes were subjected to immunoblot analysis with anti-P-Tyr and anti-PKC $\delta$  (top two panels). The immune complexes were also incubated with [ $\gamma$ - $^{32}$ P]ATP alone (middle panel) and in the presence of histone H1 or His-SHPTP1 (bottom two panels). Reaction products were separated by SDS-PAGE and analyzed by using autoradiography.



and autoradiography demonstrated phosphorylation of SHPTP1 by PKC $\delta$  CF (Fig. 3A, left). GST-PKC $\delta$  CF was also incubated with histone H1 to document activity of fusion protein (Fig. 3A, right). To further define whether SHPTP1 is a substrate for PKC $\delta$ , studies were performed with biologically active full-length recombinant PKC $\delta$  (Calbiochem). The recombinant PKC $\delta$  exhibited constitutive activity in autophosphorylation assays (Fig. 3B, left). Moreover, the addition of PKC activators (phorbol 12-myristate 13-acetate, phosphatidyl serine, and lipids) resulted in only a 2-fold increase in autophosphorylation (Fig. 3B, top left). In accord with these findings, recombinant PKC $\delta$  was active in the phosphorylation of histone H1 without cofactors, and this activity was increased 4.8-fold by adding cofactors (Fig. 3B, lower and middle left). Moreover, incubation of recombinant PKC $\delta$  with His-SHPTP1 and [ $\gamma$ - $^{32}$ P]ATP demonstrated that SHPTP1 is a PKC $\delta$  substrate (Fig. 3B, right). The results demonstrate that PKC $\delta$  phosphorylates SHPTP1 in vitro (Fig. 3B). To assess whether endogenous PKC $\delta$  phosphorylates SHPTP1 in the cellular response to DNA damage, anti-PKC $\delta$  immunoprecipitates from U-937 cells were subjected to immunoblot analysis with anti-P-Tyr and anti-PKC $\delta$ . The results demonstrate that ara-C treatment is associated with increased tyrosine phosphorylation of PKC $\delta$  and no detectable effect on PKC $\delta$  levels (Fig. 3C). Anti-PKC $\delta$  immunoprecipitates were also analyzed by incubation with [ $\gamma$ - $^{32}$ P]ATP alone and in the presence of histone H1 or SHPTP1. Analysis of the reaction products demonstrated that ara-C treatment is associated with the induction of PKC $\delta$  activity (Fig. 3C).

**PKC $\delta$  Down-Regulates SHPTP1 Tyrosine Phosphatase Activity.** To assess the functional significance of the interaction between SHPTP1 and PKC $\delta$  in vitro, GST-SHPTP1 was incubated with various amounts of recombinant PKC $\delta$  and synthetic phosphopeptides as substrates. The results demonstrate that the tyrosine phosphatase activity of SHPTP1 is inhibited by PKC $\delta$  in a concentration-dependent manner (Fig. 4). To investigate the tyrosine phosphatase activity of SHPTP1 in response to DNA damage, U-937/neo and U-937/Bcl-x<sub>L</sub> cells were treated with ara-C and harvested at 1, 2, 4, and 6 h. Lysates from control or ara-C-

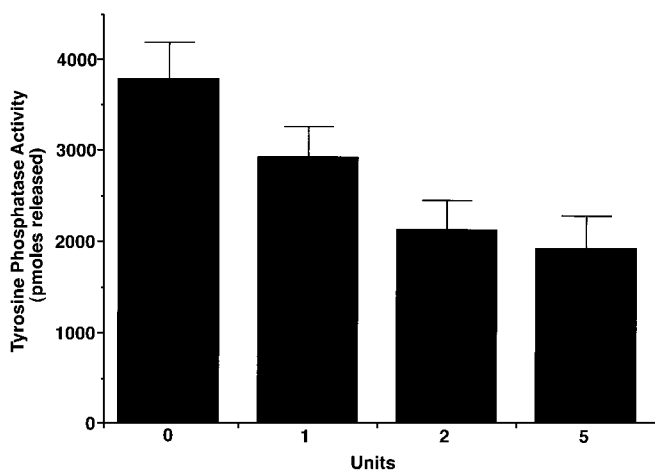
treated cells were subjected to immunoprecipitation with anti-SHPTP1, and the precipitates were analyzed for dephosphorylation of the synthetic phosphopeptide. The results demonstrate that, although SHPTP1 activity is transiently stimulated at 1 and 2 h, ara-C-induced decreases in SHPTP1 activity were observed at 4 and 6 h of treatment (Fig. 5A). Similar findings were obtained in both ara-C-treated U-937/neo and U-937/Bcl-x<sub>L</sub> cells. As shown previously, U-937/Bcl-x<sub>L</sub> cells are resistant to ara-C-induced apoptosis (Datta et al., 1995). Thus, the decrease in SHPTP1 activity is not attributable to ara-C-induced cytotoxicity. To determine whether PKC $\delta$  contributes to down-regulation of SHPTP1 activity, U-937 cells were pretreated with the PKC $\delta$  inhibitor rottlerin (Gschwendt et al., 1994) for 30 min followed by ara-C treatment for 4 h. The results demonstrate that rottlerin attenuates ara-C-induced activation of PKC $\delta$  (Fig. 5B). In contrast, there was no detectable effect of the PKC $\alpha$  and - $\beta$  inhibitor G66976 (Fig. 5B) (Martiny-Baron et al., 1993). Rottlerin, but not G66976, also inhibited ara-C-induced down-regulation of SHPTP1 (Fig. 5C). These findings provide support for the involvement of PKC $\delta$  in the down-regulation of SHPTP1 activity in response to DNA damage.

To confirm the interaction between SHPTP1 and PKC $\delta$ , 293T cells were cotransfected with Flag-SHPTP1 and GFP-vector, GFP-PKC $\delta$  CF, or GFP-PKC $\delta$  CF(K-R). At 36 h after transfection, cells were left untreated or were treated with ara-C for 4 h. An analysis of anti-Flag immunoprecipitates demonstrated that the tyrosine phosphatase activity of SHPTP1 is significantly decreased in GFP-PKC $\delta$  CF transfected cells compared with that found in control cells (Fig. 6). In contrast, ara-C-mediated down-regulation of SHPTP1 activity was abrogated in cells transfected with kinase-inactive GFP-PKC $\delta$  CF(K-R) (Fig. 6). These results indicate that the kinase activity of PKC $\delta$  is required in part for down-regulation of SHPTP1 activity in response to DNA damage.

## Discussion

The mechanisms by which genotoxic stress is converted into intracellular signals that regulate protein tyrosine phosphorylation are for the most part unknown. Insights have been derived from the findings that c-Abl and Lyn are activated by agents that induce DNA damage and apoptosis (Kharbanda et al., 1994, 1995b; Yoshida et al., 1999; Yoshida et al., 2000). c-Abl phosphorylates and activates the SHPTP1 tyrosine phosphatase in response to DNA damage (Kharbanda et al., 1996). In turn, the activation of SHPTP1 down-regulates c-Abl-mediated signaling (Kharbanda et al., 1996). Other studies have shown that Lyn stimulates the tyrosine phosphatase activity of SHPTP1 and that, in a potential feedback mechanism, SHPTP1 inhibits Lyn activity (Yoshida et al., 1999). Activation of c-Abl and Lyn by DNA damaging agents signals the induction of stress-activated protein kinase activity (Yoshida et al., 2000). By contrast, the stress-activated protein kinase signaling pathway is subject to down-regulation by SHPTP1 after DNA damage (Kharbanda et al., 1996). These findings have collectively supported a role for SHPTP1 in regulating protein tyrosine phosphorylation induced in the response to genotoxic stress.

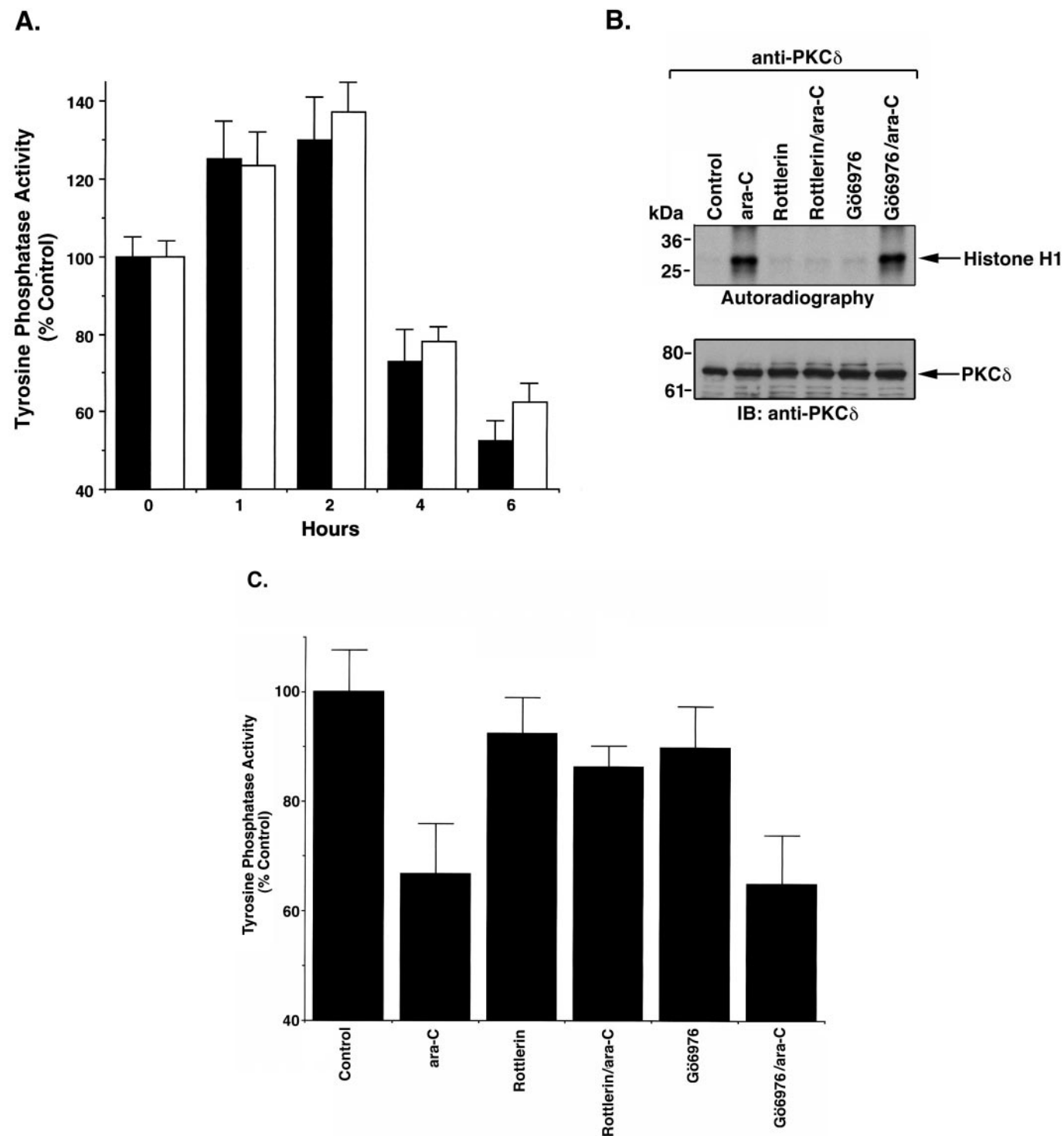
ara-C induces DNA double-strand breaks by incorporating into replicating DNA and functioning as a relative chain terminator (Kufe et al., 1980; Ohno et al., 1988). The cellular



**Fig. 4.** PKC $\delta$  down-regulates SHPTP1 activity in vitro. GST-SHPTP1 was incubated with the phosphopeptides and the indicated units of recombinant PKC $\delta$  for 30 min. The results are expressed in picomoles of phosphate released (mean  $\pm$  S.D. of two independent experiments each performed in triplicate).

response to ara-C involves the induction of early-response gene expression (Kharbanda et al., 1990, 1993) and apoptosis (Gunji et al., 1991). Treatment with ara-C is also associated

with the activation of both serine/threonine (Kharbanda et al., 1992, 1993; Saleem et al., 1995) and tyrosine (Kharbanda et al., 1995a; Yuan et al., 1995) kinases. The present study



**Fig. 5.** Treatment of ara-C is associated with the attenuation of SHPTP1 activity. A, U-937/neo (■) and U-937/Bcl-x<sub>L</sub> (□) cells were treated with 10  $\mu$ M ara-C for the indicated times. Lysates were subjected to immunoprecipitation with anti-SHPTP1. Immune complexes were incubated with the phosphopeptide as a substrate. The data represent the percentage of control of tyrosine phosphatase activity. The results are expressed as the mean  $\pm$  S.D. of three independent experiments. B, U-937 cells were treated with rottlerin or Gö6976 for 30 min followed by the ara-C treatment for 4 h. Anti-PKC $\delta$  immunoprecipitates were incubated with histone H1 and [ $\gamma$ -<sup>32</sup>P]ATP. Reaction products were separated by SDS-PAGE and analyzed by autoradiography (top). Cell lysates were analyzed by immunoblotting with anti-PKC $\delta$  (lower bottom). C, Anti-SHPTP1 immunoprecipitates as prepared in B were assayed for tyrosine phosphatase activity. The data represent the percentage of control of tyrosine phosphatase activity (mean  $\pm$  S.D. of three independent experiments). Values obtained for ara-C-treated and Gö6976/ara-C-treated, but not rottlerin/ara-C-treated, U-937 cells were significantly different from the control values (*t* test; *p* < 0.05).

extends these findings by demonstrating that ara-C induces PKC $\delta$  activity. As shown for ionizing radiation (Yuan et al., 1998), activation of PKC $\delta$  in ara-C-treated cells is signaled, at least in part, by c-Abl-dependent tyrosine phosphorylation (K. Yoshida and D. Kufe, unpublished observations). In this context, although ara-C activates c-Abl (Kharbanda et al., 1995a), c-Abl confers activation of PKC $\delta$  in the absence of lipid cofactors (Konishi et al., 1997; Sun et al., 2000). c-Abl-mediated phosphorylation of PKC $\delta$  on Tyr512 in the activation loop has been shown to be sufficient for the induction of PKC $\delta$  activity (Sun et al., 2000).

Previous work has shown that c-Abl associates constitutively with both PKC $\delta$  (Yuan et al., 1998) and SHPTP1 (Kharbanda et al., 1996). Other studies have demonstrated that c-Abl and PKC $\delta$  are present in a complex with the DNA-dependent protein kinase and that both c-Abl and PKC $\delta$  are functional in the down-regulation of DNA-dependent protein kinase activity (Bharti et al., 1998). The present study demonstrates that PKC $\delta$  also functions in the regulation of SHPTP1. The results support a direct interaction between SHPTP1 and the PKC $\delta$  C-terminal catalytic domain. Moreover, the results demonstrate PKC $\delta$ -mediated phosphorylation of SHPTP1 in vitro and in cells treated with ara-C. Phosphorylation of SHPTP1 on serine and/or threonine has been reported in the response of cells to stimuli other than DNA damage (Lorenz et al., 1994; Zhao et al., 1994). However, to our knowledge, there is no available in-

formation regarding which serine/threonine kinases are responsible for phosphorylating SHPTP1. The present findings indicate that SHPTP1 is a substrate for PKC $\delta$  in the response to DNA damage.

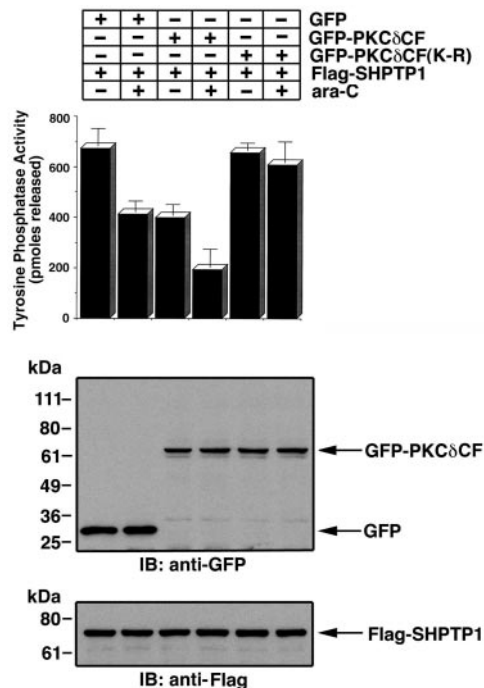
The functional significance of the interaction between PKC $\delta$  and SHPTP1 is supported by the in vitro finding that PKC $\delta$  down-regulates SHPTP1 activity. Thus, although c-Abl and Lyn activate SHPTP1 in cells exposed to DNA-damaging agents (Kharbanda et al., 1996; Yoshida et al., 1999), PKC $\delta$  activation would be expected to oppose this response. Indeed, the treatment of cells with ara-C was associated with an initial stimulation of SHPTP1 activity and then, in concert with increases in PKC $\delta$  activity, down-regulation of the phosphatase function. The demonstration that rottlerin attenuates the down-regulation of SHPTP1 activity provided support for involvement of PKC $\delta$ . Additional support was obtained from the demonstration that expression of the kinase-inactive PKC $\delta$  CF(K-R) mutant also blocks down-regulation of SHPTP1 in response to ara-C treatment. These findings thus support a model in which ara-C-induced DNA damage activates a signaling pathway that involves a novel functional interaction between a serine/threonine kinase and a tyrosine phosphatase.

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**Fig. 6.** Kinase activity of PKC $\delta$  is required for the down-regulation of SHPTP1 in response to DNA damage. 293T cells were cotransfected with Flag-SHPTP1 and GFP, GFP-PKC $\delta$  CF, or GFP-PKC $\delta$  CF(K-R). At 36 h after transfection, cells were left untreated or were treated with 10  $\mu$ M ara-C for 4 h. Cell lysates were subjected to immunoprecipitation with anti-Flag. Immune complexes were analyzed for tyrosine phosphatase activity. The results are expressed in picomoles of phosphate released (mean  $\pm$  S.D. of three independent experiments) (top). Values obtained for PKC $\delta$  CF-transfected, but not PKC $\delta$  CF(K-R)-transfected, 293T cells were significantly different from the control values ( $t$  test;  $p < 0.05$ ). Cell lysates were also subjected to immunoblotting with anti-GFP (middle) or anti-Flag (bottom).

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