Negative Regulation of the SHPTP1 Protein Tyrosine Phosphatase by Protein Kinase C δ 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 δ (PKCδ) associates with SHPTP1. The PKCδ catalytic domain binds directly to SHPTP1. The results also demonstrate that PKCδ is required, at least in part, for phosphorylation and inactivation of SHPTP1. The phosphatase activity of SHPTP1 was attenuated by coinubcation with PKCδ in vitro. In addition, treatment of U-937 human myeloid leukemia cells with 1-β-D-arabinofuranosylcytosine (ara-C) was associated with induction of the PKCδ kinase function and inhibition of SHPTP1 activity. Down-regulation of SHPTP1 by ara-C was blocked by the PKCδ inhibitor rottlerin but not by the PKCα and -β inhibitor Go6976. Moreover, transient coexpression studies with a dominant-negative mutant of PKCδ demonstrate that the kinase activity of PKCδ is required for the down-regulation of SHPTP1. These findings support the functional interaction between PKCδ 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. PKCδ 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 PKCδ 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 PKCδ is constitutively active and induces apoptosis in various cell lines (Ghayur et al., 1996). These findings support a role for

ABBREVIATIONS: PKCδ, protein kinase C δ; ara-C, 1-β-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-PKCδ, green fluorescent protein-tagged PKCδ.
PKCδ in the apoptotic response to genotoxic stress (Emoto et al., 1995, 1996; Ghayur et al., 1996). This study demonstrates that PKCδ interacts with SHPTP1. The results show that the C-terminal catalytic fragment of PKCδ is responsible for direct binding to SHPTP1. We also show that phosphorylation of SHPTP1 by PKCδ is associated with the down-regulation of SHPTP1 tyrosine phosphatase activity. The results also demonstrate that activation of PKCδ 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-xL (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 µg/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 µM ara-C (Sigma-Aldrich, St. Louis, MO), 10 µM rottlerin (Calbiochem, San Diego, CA), or 50 nM Go6976 (Calbiochem).

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**Fig. 1.** Association of SHPTP1 with PKCδ. A and B, 293T cells were transfected with Flag-SHPTP1 and/or GFP-PKCδ. 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 µM ara-C and harvested at 1 h. Lysates from control and treated cells were immunoprecipitated with preimmune rabbit serum (PIRS), anti-PKCδ (C), or anti-SHPTP1 (D). The precipitates were subjected to immunoblotting with anti-SHPTP1 and anti-PKCδ.
In Vitro Binding Assays. Glutathione S-transferase (GST) and GST-SHPTP1 (2 μg) were incubated with full-length recombinant PKCδ (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 analyzed by using immunoblotting with anti-PKCδ antibody. In reciprocal experiments, GST, GST-PKCδ full-length (FL), GST-PKCδ regulatory domain (RD), and GST-PKCδ CF (Bharti et al., 1998) (2 μg 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 PKCδ was prepared from insect Sf-9 cells infected with a baculovirus containing the PKCδ cDNA (Calbiochem). The recombinant PKCδ preparation contains phospholipids and has a specific activity of >800 units/mg (according to the manufacturer). One unit of PKCδ activity is defined as the amount of enzyme that transfers 1.0 nmol of phosphate to the PKCδ peptide substrate per minute at 37°C. GST-SHPTP1 (5 μg) or His-SHPTP1 (2 μg) proteins were incubated in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, 2 mM DTT, and 0.1 mM sodium vanadate) with GST-PKCδ CF or recombinant PKCδ and [γ-32P]ATP (3000 Ci/mmol; PerkinElmer Life Science Products) for 15 min at 30°C. Alternatively, PKCδ 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, PKCδ kinase assays were also performed in the presence of PKC activators (10 μM phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidyl serine, and Triton X-100 mixed micelles; Invitrogen, Carlsbad, CA). For the analysis of PKCδ activity in the anti-PKCδ 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 [γ-32P]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 (RLIE-DAEYAAPARG) 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
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 PKCδ.** To assess potential interactions between SHPTP1 and PKCδ in cells, lysates from 293T cells cotransfected with Flag-tagged SHPTP1 (Flag-SHPTP1) and GFP-tagged PKCδ (GFP-PKCδ) 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 PKCδ (Fig. 1B). To determine whether endogenous SHPTP1 associates with endogenous PKCδ, anti-PKCδ immunoprecipitates from human U-937 cells were subjected to immunoblotting with anti-SHPTP1. The results demonstrate that PKCδ associates with SHPTP1 from both control and ara-C–treated cells (Fig. 1C).

**PKCδ Phosphorylates SHPTP1.** To determine whether SHPTP1 is phosphorylated by PKCδ in vitro, kinase-active GST-PKCδ CF was incubated with GST-SHPTP1 and [γ-32P]ATP. GST-PKCδ 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

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**Fig. 3.** PKCδ phosphorylates SHPTP1. A, kinase-active GST-PKCδ CF was incubated with purified GST-SHPTP1 and [γ-32P]ATP (left). Histone H1 was used as a positive control (right). B, kinase-active recombinant PKCδ was incubated with [γ-32P]ATP in the absence and presence of PKC activators (top left). Histone H1 was added to similar reactions as substrate (bottom left). Recombinant PKCδ was incubated with histone H1 (middle) or column-purified His-SHPTP1 (right) and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. C, U-937 cells were treated with 10 μM ara-C for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-PKCδ. Immune complexes were subjected to immunoblot analysis with anti-P-Tyr and anti-PKCδ (top two panels). The immune complexes were also incubated with [γ-32P]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 PKCδ CF (Fig. 3A, left). GST-PKCδ 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 PKCδ, studies were performed with biologically active full-length recombinant PKCδ (Calbiochem). The recombinant PKCδ exhibited constitutive activity in auto-phosphorylation 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 PKCδ 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 PKCδ with His-SHPTP1 and \([\gamma^{32P}]ATP\) demonstrated that SHPTP1 is a PKCδ substrate (Fig. 3B, right). The results demonstrate that PKCδ phosphorylates SHPTP1 in vitro (Fig. 3B). To assess whether endogenous PKCδ phosphorylates SHPTP1 in the cellular response to DNA damage, anti-PKCδ immunoprecipitates from U-937 cells were subjected to immunoblot analysis with anti-P-Tyr and anti-PKCδ. The results demonstrate that ara-C treatment is associated with increased tyrosine phosphorylation of PKCδ and no detectable effect on PKCδ levels (Fig. 3C). Anti-PKCδ immunoprecipitates were also analyzed by incubation with \([\gamma^{32P}]ATP\) 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 PKCs activity (Fig. 3C).

**PKCδ Down-Regulates SHPTP1 Tyrosine Phosphatase Activity.** To assess the functional significance of the interaction between SHPTP1 and PKCδ in vitro, GST-SHPTP1 was incubated with various amounts of recombinant PKCδ and synthetic phosphopeptides as substrates. The results demonstrate that the tyrosine phosphatase activity of SHPTP1 is inhibited by PKCδ 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-xL 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-xL cells. As shown previously, U-937/Bcl-xL 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 PKCδ contributes to down-regulation of SHPTP1 activity, U-937 cells were pretreated with the PKCδ inhibitor rottlerin (Geschwendt 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 PKCδ (Fig. 5B). In contrast, there was no detectable effect of the PKCα and β inhibitor Gö6976 (Fig. 5B) (Martin-Baron et al., 1993). Rottlerin, but not Gö6976, also inhibited ara-C–induced down-regulation of SHPTP1 (Fig. 5C). These findings provide support for the involvement of PKCδ in the down-regulation of SHPTP1 activity in response to DNA damage.

To confirm the interaction between SHPTP1 and PKCδ, 293T cells were cotransfected with Flag-SHPTP1 and GFP-vector, GFP-PKCδ CF, or GFP-PKCδ 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-PKCδ 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-PKCδ CF(K-R) (Fig. 6). These results indicate that the kinase activity of PKCδ 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
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

![Graph A](image)

**Fig. 5.** Treatment of ara-C is associated with the attenuation of SHPTP1 activity. A, U-937/neo (■) and U-937/Bcl-xL (□) cells were treated with 10 μM 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 ± S.D. of three independent experiments. B, U-937 cells were treated with rottlerin or Go6976 for 30 min followed by the ara-C treatment for 4 h. Anti-PKCδ immunoprecipitates were incubated with histone H1 and [γ-32P]ATP. Reaction products were separated by SDS-PAGE and analyzed by autoradiography (top). Cell lysates were analyzed by immunoblotting with anti-PKCδ (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 ± S.D. of three independent experiments). Values obtained for ara-C–treated and Go6976/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 PKCδ activity. As shown for ionizing radiation (Yuan et al., 1998), activation of PKCδ in ara-C–treated cells is signaled, at least in part, by c-Ab−dependent tyrosine phosphorylation (K. Yoshida and D. Kufe, unpublished observations). In this context, although ara-C activates c-Ab (Kharbanda et al., 1993a), c-Ab confers activation of PKCδ in the absence of lipid cofactors (Konishi et al., 1997; Sun et al., 2000). c-Ab–mediated phosphorylation of PKCδ on Tyr512 in the activation loop has been shown to be sufficient for the induction of PKCδ activity (Sun et al., 2000).

Previous work has shown that c-Ab associates constitutively with both PKCδ (Yuan et al., 1998) and SHPTP1 (Kharbanda et al., 1996). Other studies have demonstrated that c-Ab and PKCδ are present in a complex with the DNA-dependent protein kinase and that both c-Ab and PKCδ are functional in the down-regulation of DNA-dependent protein kinase activity (Bharti et al., 1998). The present study demonstrates that PKCδ also functions in the regulation of SHPTP1. The results support a direct interaction between SHPTP1 and the PKCδ C-terminal catalytic domain. Moreover, the results demonstrate PKCδ–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-

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

The functional significance of the interaction between PKCδ and SHPTP1 is supported by the in vitro finding that PKCδ down-regulates SHPTP1 activity. Thus, although c-Ab and Lyn activate SHPTP1 in cells exposed to DNA-damaging agents (Kharbanda et al., 1996; Yoshida et al., 1999), PKCδ 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 PKCδ activity, down-regulation of the phosphatase function. The demonstration that rotellin attenuates the down-regulation of SHPTP1 activity provided support for involvement of PKCδ. Additional support was obtained from the demonstration that expression of the kinase-inactive PKCδ 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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References


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