The Lyn protein-tyrosine kinase is activated in the cellular response to DNA-damaging agents. Here we demonstrate that Lyn associates constitutively with the SHPTP1 protein-tyrosine phosphatase. The SH3 domain of Lyn interacts directly with SHPTP1. The results show that Lyn phosphorylates SHPTP1 at the C-terminal Tyr-564 site. Lyn-mediated phosphorylation of SHPTP1 stimulates SHPTP1 tyrosine phosphatase activity. We also demonstrate that treatment of cells with 1-β-D-arabinofuranosylcytosine and other genotoxic agents induces Lyn-dependent phosphorylation and activation of SHPTP1. The significance of the Lyn-SHPTP1 interaction is supported by the demonstration that activation of Lyn contributes in part to the apoptotic response to ara-C treatment and that SHPTP1 attenuates this response. These findings support a functional interaction between Lyn and SHPTP1 in the response to DNA damage.

The response of mammalian cells to DNA damage includes cell cycle arrest, activation of DNA repair, and induction of apoptosis. The signaling events responsible for regulation of the genotoxic stress response, however, are largely unknown. Certain insights have been derived from the finding that DNA-damaging agents activate a nuclear complex that consists in part of the c-Abl and Lyn protein-tyrosine kinases. c-Abl associates with the DNA-dependent protein kinase (DNA-PK) and is activated in the response to DNA damage by a DNA-PK-dependent mechanism (1–3). The ataxia telangiectasia mutated (ATM) gene product also associates with c-Abl and contributes to c-Abl activation (4, 5). The demonstration that c-Abl binds to p53, induces the transactivation function of p53, and activates p21 expression has supported involvement of c-Abl in regulation of the p53-dependent G1 arrest response (6–8). Other studies have demonstrated that c-Abl interacts with the p73 homolog of p53 in the apoptotic response to DNA damage (9). The interaction of c-Abl and the Rad51 protein has also provided support for involvement of c-Abl in recombinational repair of DNA strand breaks (10). In addition, a proapoptotic function for c-Abl in the response to DNA damage has been attributed to negative regulation of phosphatidylinositol 3-kinase and induction of the stress-activated protein kinase pathway (1, 2, 11, 12). These findings have supported a role for c-Abl in the growth arrest, DNA repair, and apoptotic responses to genotoxic stress.

The Lyn tyrosine kinase is a member of the Src family that contains SH2 and SH3 domains and an N-terminal sequence, which when myristoylated, serves as a membrane localization signal (13). Cell fractionation and confocal microscopy studies have also demonstrated expression of Lyn in the nucleus (14). Nuclear Lyn is activated by DNA damage associated with exposure to 1-β-D-arabinofuranosylcytosine (ara-C), ionizing radiation, and certain alkylating agents (15–17). Lyn, like c-Abl, interacts with the DNA-PK complex (18). The interaction between Lyn and DNA-PK catalytic subunit (DNA-PKcs) from Ku/DNA complexes after repair to permit relocation at new sites of DNA damage (18). The activation of nuclear Lyn by DNA damage is also associated with binding of Lyn to Cdc2 (14–17). Lyn phosphorylates Cdc2 on Tyr-15 and inhibits Cdc2 activity (14–17). As activation of Cdc2 in a complex with cyclin B is required for the transition of cells from G2 to M phase (19), Lyn may function as an effector of G2/M regulation in response to DNA damage. Other studies have demonstrated that the arrest of cells at G1/S phase by ara-C treatment is associated with binding of activated Lyn to Cdk2 (20). These findings collectively support a role for nuclear Lyn in the response of cells to DNA damage.

The present studies have addressed the involvement of other Lyn-associated signals in the DNA damage response. The results demonstrate that treatment with ara-C induces Lyn-dependent phosphorylation of the SHPTP1 protein-tyrosine phosphatase (21, 22). We show that Lyn activates SHPTP1, and in a potential feedback mechanism, SHPTP1 down-regulates the Lyn kinase function. The results also demonstrate that SHPTP1 attenuates Lyn-dependent induction of apoptosis in the response to DNA damage.

MATERIALS AND METHODS

Cell Culture—Human U-937 myeloid leukemia cells (ATCC, Manassas, VA) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. 293T embryonal kidney and HeLa cells were grown in DMEM containing 10% fetal bovine serum and antibiotics. Cells were treated with the indicated concentrations of ara-C (Sigma). Irradiation was performed at room temperature using a Gammasell 1000 (Atomic Energy of Canada, Ottawa, Ontario, Canada) under aerobic conditions with a 173Cs source emitting at a fixed dose rate of 0.76 gray/min as determined by dosimetry.

Cell Transfections—The Lyn(K-R) cDNA that encodes the kinase-negative mutant (the lysine residue at position 275 in the putative ATP-binding site replaced with arginine) and the SHPTP1 mutants were generated by site-directed mutagenesis. Mutations were confirmed by sequencing. Lyn wild-type and mutant cDNAs were subcloned into pSRR (23) and pEP2 (24) vectors.

U-937 cells were resuspended at 10^6/ml and transfected with empty pEP2 vector or pEP2/Lyn(K-R) by electroporation (Gene Pulser; Bio-
Rad; 0.25 V, 960 microfarads). HeLa cells were transfected with pSRα vector or pSRα/Lyn (K-R) by electroporation. Two days posttransfection, the cells were cultured in media containing 400 μg/ml geneticin sulfate (Life Technologies, Inc.), and individual clones were selected by limiting dilution. 293T cells were transiently transfected with pSRα/Lyn, pSRα/Lyn-K(R), pDNA3/SHPTP1, pDNA3/SHPTP1(C-S) (25), pDNA3/SHPTP1(Y564F), or pDNA3/SHPTP1(C-S/Y564F) by the calcium phosphate method. At 48 h posttransfection, cells were harvested for preparing whole cell lysates. HeLa/neo cells or HeLa/Lyn (K-R) cells were transiently cotransfected with 5 μg of pEGFP-C1 vector (CLONTECH) and 10 μg of pCDNA3 vector, pCDNA3/SHPTP1, pCDNA3/SHPTP1(C-S), or pCDNA3/SHPTP1(Y564F) using SuperFect transfection reagent (Qiagen). At 48 h posttransfection, cells were left untreated or treated with 10 μM ara-C and then analyzed by flow cytometry.

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, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM sodium fluoride, and 10 μg/ml aprotinin, leupeptin, and pepstatin A). Soluble proteins were incubated with anti-Lyn antibody (sc-287; Santa Cruz Biotechnology) or anti-SHPTP1 antibody (sc-287; Santa Cruz Biotechnology) for 1 h at 4 °C followed by 1 h of incubation with protein A-Sepharose beads (Amersham Pharmacia Biotech). The immune complexes were washed three times with lysis buffer, resuspended in 30% SDS-acrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose filters. The residual binding sites were blocked by incubating with 5% dry milk in PBS, 0.05% Tween 20 overnight at 4 °C. The filters were incubated with anti-Lyn (mouse monoclonal antibody; Transduction Laboratory), anti-SHPTP1, or anti-phosphotyrosine antibody (4G10; Upstate Biotechnology Inc.) for 1 h with shaking. After washing twice with 5% dry milk in PBS, 0.05% Tween 20, the filters were incubated with anti-rabbit or anti-mouse IgG peroxidase conjugate (Amersham Pharmacia Biotech). The antigen-antibody complexes were visualized by chemiluminescence (NEN Life Science Products).

Fusion Protein Binding Assays—Glutathione S-transferase (GST), GST-Lyn (1–243), GST-Lyn (1–131), GST-Lyn (30–130), and GST-Lyn (131–243) (26) were purified by affinity chromatography using glutathione-Sepharose beads (Amersham Pharmacia Biotech). The immune complexes were washed three times with lysis buffer, resuspended in 30% SDS-acrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose filters. The residual binding sites were blocked by incubating with 5% dry milk in PBS, 0.05% Tween 20 overnight at 4 °C. After washing twice with 5% dry milk in PBS, 0.05% Tween 20, the filters were incubated with anti-rabbit or anti-mouse IgG peroxidase conjugate (Amersham Pharmacia Biotech). The antigen-antibody complexes were visualized by chemiluminescence (NEN Life Science Products).

RESULTS AND DISCUSSION

The findings that c-Abl forms a nuclear complex with Lyn (27) and that c-Abl interacts with SHPTP1 (25) prompted studies on a potential interaction between Lyn and SHPTP1. To determine whether Lyn associates with SHPTP1, U-937 cells were subjected to immunoprecipitation with anti-Lyn. Immunoblot analysis of the anti-Lyn immunoprecipitates with anti-SHPTP1 demonstrated reactivity at 70 kDa (Fig. 1A). Similar findings were obtained in cells treated with ara-C (Fig. 1A). In a reciprocal experiment, analysis of anti-SHPTP1 immunoprecipitates with anti-Lyn confirmed a constitutive association of these proteins (Fig. 1B). Binding of Lyn and SHPTP1 was assessed by incubating U-937 cell lysates with GST fusion proteins prepared with Lyn fragments. Analysis of the adsortates with anti-SHPTP1 demonstrated binding to Lyn (1–243), Lyn (1–131), and Lyn (30–130) but not Lyn (131–243) (Fig. 1C).

FIG. 1. Direct association of Lyn and SHPTP1. U-937 cells were treated with 10 μM ara-C and harvested at 1 h. Lysates from control and treated cells were subjected to immunoprecipitation with preimmune serum (PIRS), anti-Lyn (A), or anti-SHPTP1 (B). The proteins were resolved and analyzed by immunoblotting (IB) with anti-SHPTP1 or anti-Lyn. C, U-937 cell lysate was incubated with GST, GST-Lyn (1–243), GST-Lyn (1–131), GST-Lyn (30–130), and GST-Lyn (131–243) fusion proteins. The adsortates were analyzed by immunoblotting (IB) with anti-SHPTP1. D, recombinant Lyn was incubated with glutathione beads containing GST or GST-SHPTP1. The adsortates were analyzed by immunoblotting (IB) with anti-Lyn.

(Becton Dickinson). The numbers of cells positive for green fluorescence with sub-G1 DNA content were determined with a CELLQuest program (Becton Dickinson).
As Lyn (30–130) contains the SH3 and not the SH2 domain, these findings suggest that Lyn SH3 is responsible for binding to SHPTP1. To determine whether the interaction is direct, glutathione beads containing GST or GST-SHPTP1 were incubated with purified Lyn protein. The beads were washed, and

**Fig. 2. Phosphorylation of SHPTP1 by Lyn.** A, recombinant Lyn was incubated with GST or GST-SHPTP1 and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. B, recombinant Lyn was incubated with purified His-tagged wild-type (wt), mutant Y536F, Y541F, or Y646F SHPTP1 proteins (2 μg each) in kinase buffer for 15 min at 30 °C. The reaction products were analyzed by SDS-PAGE and autoradiography. C, 293T cells were cotransfected with 3 μg of SHPTP1(C-S) or SHPTP1(C-S/Y564F) and 3 μg of pSRα (vector), Lyn, or Lyn(K-R). Anti-SHPTP1 immunoprecipitates (IP) were analyzed by immunoblotting (IB) with anti-Tyr(P) (upper panel) or anti-SHPTP1 (middle panel). Lysates were also subjected to immunoblotting (IB) with anti-Lyn (lower panel).

**Fig. 3. Lyn activates SHPTP1 tyrosine phosphatase activity.** A, GST-SHPTP1 was incubated with the phosphopeptide and buffer (●) or 1 unit (○) or 5 units (▲) of recombinant Lyn. Activity is expressed as pmol of phosphate released. The results are representative for the mean ± S.D. of three independent experiments, each performed in triplicate. B, 293T cells were cotransfected with 3 μg of SHPTP1 and 5 μg of pSRα (vector), Lyn, or Lyn(K-R). Anti-SHPTP1 immunoprecipitates were incubated with the phosphopeptide as a substrate for 30 min. Activity is expressed as pmol of phosphate released. The results represent the mean ± S.D. of three independent experiments. wt, wild type. C, 293T cells were transfected with the indicated amounts (μg) of pcDNA3 (vector), Lyn, SHPTP1, or SHPTP1(C-S). Cell lysates were immunoprecipitated with anti-Lyn. The immunoprecipitates (IP) were incubated with [γ-32P]ATP (upper panel) or with enolase and [γ-32P]ATP (second panel). Reaction products were resolved by 10% SDS-PAGE and analyzed by autoradiography (upper two panels). Lysates were also subjected to immunoblot (IB) analysis with anti-Lyn or anti-SHPTP1 (lower two panels).
protein was eluted by boiling in SDS. Analysis of the eluted protein by immunoblotting with anti-Lyn demonstrated a direct interaction between Lyn and SHPTP1 (Fig. 1D).

To determine whether Lyn phosphorylates SHPTP1 in vitro, DNA damage. A, U-937/neo and U-937/Lyn(K-R) cells were treated with 10\(^{-6}\) or 10\(^{-5}\) M ara-C and harvested at 1 h. Anti-Lyn immunoprecipitates (IP) were incubated with enolase and \(\gamma\)-\(\text{ATP}\). Reaction products were analyzed by SDS-PAGE and autoradiography. B, U-937/neo and U-937/Lyn(K-R) cells were treated with 10\(^{-5}\) M ara-C or 20-Gy ionizing radiation (IR) and collected after 1 h. Cell lysates from control and treated cells were subjected to immunoprecipitation with anti-SHPTP1. Immune complexes were analyzed by immunoblotting (IB) with anti-Tyr(P) (upper panel) or anti-SHPTP1 (lower panel). C, U-937/neo (solid bars) and U-937/Lyn(K-R) (open bars) cells were treated with 10\(^{-5}\) M ara-C and collected after 1 h or 2 h. Cell lysates from control and ara-C-treated cells were subjected to immunoprecipitation with anti-SHPTP1. SHPTP1 tyrosine phosphatase assays were performed using the synthetic phosphopeptide as substrate. The data represent the percentage increase in tyrosine phosphatase activity compared with untreated controls. The results are expressed as the mean ± S.D. of three independent experiments. Values obtained for the U-937/neo and U-937/Lyn(K-R) cells were significantly different (t test; \(p < 0.05\)) at both time points.
To determine whether Lyn activates SHPTP1 in vivo, 293T cells were cotransfected with kinase-active Lyn or Lyn(K-R) and phosphatase-active SHPTP1. Anti-SHPTP1 immunoprecipitates were analyzed for tyrosine phosphatase activity. The results demonstrate that Lyn and not Lyn(K-R) stimulates SHPTP1 activity (Fig. 3B). As a control, anti-SHPTP1 immunoprecipitates from cells transfected with SHPTP1(C-S) exhibited little if any phosphatase activity (data not shown). These findings demonstrate that Lyn stimulates SHPTP1 activity in vitro and in vivo. In reciprocal experiments, anti-Lyn immunoprecipitates were analyzed for Lyn kinase activity. In control cells transfected with Lyn alone, the anti-Lyn immunoprecipitates exhibited autophosphorylation of Lyn and phosphorylation of enolase (Fig. 3C). Cotransfection of Lyn and 1 μg of SHPTP1 had little effect, whereas cotransfection of Lyn and 5 μg of SHPTP1 was associated with down-regulation of Lyn activity (Fig. 3C). By contrast, cotransfection of Lyn and SHPTP1(C-S) had no apparent effect on Lyn and enolase phosphorylation (Fig. 3C). These findings support a model in which Lyn stimulates the tyrosine phosphatase activity of SHPTP1, and in a potential feedback mechanism, SHPTP1 inhibits Lyn-mediated phosphorylation.

Treatment of cells with ara-C and other DNA-damaging agents is associated with induction of Lyn activity (14–17). To determine whether activation of Lyn is associated with SHPTP1 phosphorylation, we prepared U-937 cells that stably express a kinase-inactive Lyn(K-R) mutant. U-937 cells expressing the empty pEF2 vector (U-937/neo) or Lyn(K-R) were exposed to ara-C and harvested at 1 h. Anti-Lyn immunoprecipitates were analyzed for autophosphorylation and enolase phosphorylation in the presence of [γ-32P]ATP. Analysis of the reaction products by SDS-PAGE and autoradiography demonstrated induction of Lyn activity in ara-C-treated U-937/neo cells (Fig. 4A). By contrast, ara-C-induced Lyn activity was substantially abrogated in U-937/Lyn(K-R) cells (Fig. 4A). To assess Lyn phosphorylation of SHPTP1, lysates from ara-C-treated cells were subjected to immunoprecipitation with anti-SHPTP1, and the precipitates were analyzed by immunoblotting with anti-Tyr(P). The results demonstrate tyrosine phosphorylation of SHPTP1 after ara-C treatment of U-937/neo but not U-937/Lyn(K-R) cells (Fig. 4B). Similar studies were performed with irradiated cells to assess the effects of other DNA-damaging agents. The results demonstrate that ionizing radiation-induced tyrosine phosphorylation of SHPTP1 is abrogated in the cells expressing Lyn(K-R) (Fig. 4B). To determine whether DNA damage is associated with induction of SHPTP1 activity, the anti-SHPTP1 immunoprecipitates from ara-C-treated cells were analyzed for dephosphorylation of the synthetic phosphopeptide. ara-C-induced increases in SHPTP1 activity were attenuated in U-937/Lyn(K-R) cells compared with U-937/neo cells (Fig. 4C). These findings demonstrate that ara-C-induced activation of Lyn is associated with tyrosine phosphorylation of SHPTP1 and stimulation of SHPTP1 activity.

To assess the significance of the interaction between Lyn and SHPTP1 in the cellular response to DNA damage, we prepared HeLa cells that stably express the empty neo vector or the kinase-inactive Lyn(K-R) mutant (Fig. 5A). Treatment of HeLa/neo cells with ara-C was associated with an increase in the percentage with sub-G1 DNA (Fig. 5B). By contrast, ara-C-induced apoptosis was abrogated in part in the HeLa/Lyn(K-R) cells (Fig. 5B). These results support a role for Lyn in the apoptotic response to ara-C exposure. To define the role of Lyn in the context of SHPTP1, HeLa cells were transfected to express wild-type SHPTP1 or the phosphatase-inactive SHPTP1(C-S). Expression of SHPTP1, but not SHPTP1(C-S),...
attenuated ara-C-induced apoptosis of the HeLa/neo cells (Fig. 5C). In addition, expression of SHPTP1 or SHPTP1(C-S) had little if any effect on induction of ara-C-treated HeLa/Lyn(K-R) cells with sub-G1 DNA (Fig. 5C). These findings indicate that activation of Lyn contributes to the apoptotic response to DNA damage and that SHPTP1 attenuates the Lyn-mediated response.

To confirm the functional interaction between Lyn and SHPTP1, we expressed the SHPTP1(Y564F) mutant in 293T cells. Analysis of anti-SHPTP1 immunoprecipitates demonstrated that the tyrosine phosphatase activity of SHPTP1(Y564F) is comparable to that of wild-type SHPTP1 (Fig. 6A). Cotransfection of Lyn and wild-type SHPTP1 was associated with an increase in tyrosine phosphatase activity (Fig. 6A). By contrast and in concert with the demonstration that Lyn phosphorylates SHPTP1 on Tyr-564, cotransfection of Lyn and SHPTP1(Y564F) resulted in no significant activation of the tyrosine phosphatase function (Fig. 6A). In addition, whereas expression of wild-type SHPTP1 attenuated ara-C-induced apoptosis of HeLa/neo cells, transfection of SHPTP1(Y564F) was significantly less effective in abrogating the apoptotic response to ara-C (Figs. 5C and 6B). As a control, ara-C treatment of HeLa/Lyn(K-R) cells expressing SHPTP1(Y564F) resulted in a percentage of apoptotic cells similar to that obtained when these cells were transfected with pcDNA3, wild-type SHPTP1, or SHPTP1(C-S) (Figs. 5C and 6B). These findings demonstrate that the SHPTP1(Y564F) mutant is not activated by Lyn and that this mutant is less effective than wild-type SHPTP1 in attenuating Lyn-mediated apoptosis.

The finding that activation of Lyn by DNA-damaging agents contributes to the down-regulation of Cdc2 has indicated that Lyn is an effector of cell cycle progression in the response to DNA damage (14–17). Moreover, the interaction between Lyn and DNA-PK has supported a function for Lyn in the regulation of DNA repair (18). The present studies demonstrate that Lyn phosphorylates and activates SHPTP1 in response to genotoxic stress. To our knowledge, this is the first demonstration that DNA damage is associated with activation of a tyrosine phosphatase. Previous work has shown that Lyn forms a nuclear complex with c-Abl (27) and that c-Abl interacts with SHPTP1 (25). c-Abl phosphorylates SHPTP1 on the C-terminal Tyr-536 and Tyr-564 sites (25). Although the effects of c-Abl on SHPTP1 activity were not defined (25), more recent studies have demonstrated that c-Abl stimulates SHPTP1 at least in vitro. Thus, both Lyn and c-Abl are activated by DNA damage and phosphorylate SHPTP1 as a downstream effector. The present results also demonstrate that SHPTP1, in a potential feedback mechanism, down-regulates Lyn-mediated phosphorylation. The functional significance of the Lyn-SHPTP1 interaction is further supported by the findings that the activity of Lyn is responsible in part for DNA damage-induced apoptosis and that SHPTP1 attenuates the Lyn-dependent proapoptotic signals. Taken together with the previous demonstration that SHPTP1 down-regulates c-Abl-dependent activation of the stress-activated protein kinase cascade (25), the present findings support a model in which activation of SHPTP1 in the response to DNA damage functions as a negative regulator of events activated by Lyn- and c-Abl-mediated phosphorylation.

Acknowledgments—We thank John Combier for the GST-Lyn constructs, Tadashi Yamamoto for the Lyn cDNA, and Chris Walsh for the SHPTP1 cDNA and proteins.

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Functional Interaction between SHPTP1 and the Lyn Tyrosine Kinase in the Apoptotic Response to DNA Damage
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doi: 10.1074/jbc.274.49.34663

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