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# The stress response to ionizing radiation involves c-Abl-dependent phosphorylation of SHPTP1

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**ABSTRACT** **c-Abl is a nonreceptor tyrosine kinase that is activated by certain DNA-damaging agents. The present studies demonstrate that nuclear c-Abl binds constitutively to the protein tyrosine phosphatase SHPTP1. Treatment with ionizing radiation is associated with c-Abl-dependent tyrosine phosphorylation of SHPTP1. The results demonstrate that the SH3 domain of c-Abl interacts with a WPDHGVPSEP motif (residues 417–426) in the catalytic domain of SHPTP1 and that c-Abl phosphorylates C terminal Y536 and Y564 sites. The functional significance of the c-Abl–SHPTP1 interaction is supported by the demonstration that, like c-Abl, SHPTP1 regulates the induction of Jun kinase activity following DNA damage. These findings indicate that SHPTP1 is involved in the response to genotoxic stress through a c-Abl-dependent mechanism.**

Eukaryotic cells respond to ionizing radiation (IR) with cell cycle arrest, activation of DNA repair, and apoptosis. These responses are induced by direct interaction of IR with DNA or through the formation of hydroxyl radicals and superoxides (1, 2). However, the signaling cascades that are activated by IR-induced damage remain unclear. The finding that IR activates transcription of the *c-jun* and *EGR-1* early response genes (3–5) has supported involvement of nuclear signals. The nuclear factor κB and the tumor suppressor p53 are also activated by IR exposure (6, 7). IR-induced activation of these DNA binding proteins presumably represents early nuclear signals that regulate longer-term changes in gene expression, which constitute the IR response.

Several insights are available regarding protein kinases that transduce IR-induced signals in the nucleus. IR treatment is associated with induction of the mitogen-activated protein kinase and $pp90^{rsk}$ (8). Other studies have demonstrated that IR induces the stress-activated protein kinase (SAPK, Jun N-terminal kinase) (9–11). Both mitogen-activated protein kinase and SAPK can stimulate the transactivating potential of c-Jun by phosphorylation of S-63 and S-73 sites (12–14). SAPK is related to mitogen-activated protein kinase and, while unable to phosphorylate $pp90^{rsk}$, it is more active in phosphorylating c-Jun. The finding that SAPK phosphorylates Elk-1 (15) suggests that SAPK may also contribute to IR-induced *EGR-1* transcription through activation of serum response elements (5). SAPK is regulated by the SAPK/ERK kinase 1 (16, 17). SAPK/ERK kinase 1 may thus link IR-induced signals to SAPK and thereby activation of *c-jun* and *EGR-1* transcription.

Recent studies have demonstrated that IR activates the nuclear c-Abl protein tyrosine kinase (18). The finding that cells deficient in c-Abl fail to activate SAPK in response to IR-treatment has supported c-Abl as an upstream signal in this stress response. Other studies have shown that c-Abl binds to p53 *in vitro* and stimulates the transactivation function of p53 (19). Overexpression of c-Abl also induces the arrest of cells in G1 phase (20, 21). Because IR treatment is associated with G1 arrest, c-Abl activation could play a role in regulating both this response and induction of SAPK.

The present studies have addressed the involvement of other c-Abl-associated signals in the IR response. The results demonstrate that IR induces c-Abl-dependent phosphorylation of the protein tyrosine phosphatase SHPTP1 (22, 23). We also show that SHPTP1 is involved in regulation of the c-Abl → SAPK cascade in IR-treated cells.

## MATERIALS AND METHODS

**Cell Culture.** Human U-937 myeloid leukemia cells (American Type Culture Collection) were grown as described (9). $Abl^{-/-}$ (24), $Abl^{+}$ (18), and NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Irradiation was performed at room temperature using a Gammacell 1000 (Atomic Energy of Canada, Ottawa, Ontario) under aerobic conditions, with a $^{137}Cs$ source emitting at a fixed dose rate of 0.76 Gy $min^{-1}$ as determined by dosimetry.

**Preparation of Nuclear Lysates.** Cells were swelled in 2 ml of ice-cold hypotonic lysis buffer (1 mM EGTA/1 mM EDTA/10 mM β-glycerophosphate/2 mM $MgCl_2$/10 mM KCl/1 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/1 mM DTT/10 μg each of pepstatin, leupeptin, and aprotinin per ml) for 30 min and then subjected to Dounce homogenization (15–25 strokes, tight pestle A). The resulting lysate was loaded onto 1.5 ml of buffer A (1 M sucrose in hypotonic lysis buffer) and centrifuged at 1600 × *g* for 15 min to pellet nuclei. The pellet was washed and solubilized in lysis buffer containing 1% (vol/vol) Nonidet P-40.

**Immunoprecipitation and Immunoblot Analysis.** Immunoprecipitation was performed as described (9). In brief, soluble proteins (200 μg) were incubated with anti-Abl (K-12, Santa Cruz Biotechnology), anti-SHPTP1 (Upstate Biotechnology), or anti-P-Tyr (Upstate Biotechnology) for 1 h and precipitated with protein A-Sepharose for an additional 1 h. The resulting immune complexes were washed three times with lysis buffer, separated by electrophoresis in SDS/polyacrylamide gels, and then transferred to nitrocellulose paper. The residual binding sites were blocked by incubating the filters with 5% dry milk in phosphate-buffered saline and 0.05% Tween 20 for 1 h at room temperature. The filters were incubated with anti-Abl, anti-SHPTP1, or anti-P-Tyr for 1 h with shaking. After washing twice with phosphate-buffered saline and 0.05% Tween 20, the blots were incubated with anti-rabbit IgG peroxidase conjugate (Amersham). The antigen antibody com-

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Abbreviations: IR, ionizing radiation; SAPK, stress-activated protein kinase.

plexes were visualized by enhanced chemiluminescence (ECL detection system, Amersham).

**Fusion Protein Binding Assays.** GST and GST-Abl SH3 (25) were purified by affinity chromatography using glutathione-Sepharose beads and equilibrated in lysis buffer. Cell lysates were incubated with 2 μg of immobilized GST or GST-Abl SH3 for 2 h at 4°C. 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-SHPTP1 kinase antibody.

**Immune Complex Kinase Assays.** Lysates were precleared by incubation with 5 μg of rabbit anti-mouse IgG per ml for 1 h at 4°C and then for an additional 30 min after the addition of protein A-Sepharose. The supernatant was then incubated with anti-Abl and protein A-Sepharose for 2 h at 4°C. The protein complexes were washed three times with lysis buffer and once with kinase buffer (50 mM Tris, pH 7.4/10 mM $MgCl_2$/10 mM $MnCl_2$/1 mM dithiothreitol) and resuspended in kinase buffer containing 2–5 μCi of [γ-$^{32}$P]ATP per ml (3000 Ci/mmol; DuPont/NEN) and SHPTP1. The reaction mixture was incubated for 30 min at 30°C and terminated by the addition of SDS sample buffer. The proteins were analyzed by SDS/PAGE and autoradiography.

**Transient Transfections.** NIH 3T3 cells were transfected in serum-free media with lipofectamine (GIBCO/BRL). Cells ($2 \times 10^6$) were incubated with 1 μg of pEBG-SAPK (17) and 4 μg of (*i*) pcDNA3-SHPTP1 (wild-type), (*ii*) pcDNA3-SHPTP1 (C453S) mutant (23), or (*iii*) pcDNA3 vector. After incubation for 12 h, the cells were grown in media with serum for 36 h and then exposed to 20-Gy IR. Cell lysates were incubated with glutathione-Sepharose and immune complex kinase assays were performed with GST-Jun(2–100) as substrate.

## RESULTS AND DISCUSSION

Nuclear lysates from human U-937 cells were subjected to immunoprecipitation with anti-Abl antibody, and silver staining of the immunoprecipitate revealed the presence of a 70-kDa protein (data not shown). Immunoblot analysis of the anti-Abl immunoprecipitate with an anti-SHPTP1 antibody demonstrated reactivity at 70 kDa (Fig. 1*A*). Similar findings were obtained in cells exposed to 20-Gy IR (Fig. 1*A*). In the reciprocal experiment, analysis of anti-SHPTP1 immunoprecipitates with anti-Abl confirmed a constitutive association between these two proteins (Fig. 1*B*). Analysis of anti-SHPTP1 immunoprecipitates with anti-P-Tyr further demonstrated IR-dependent increases in tyrosine phosphorylation of SHPTP1 (Fig. 1*C*). Recent studies have shown that nuclear c-Abl is activated in cells exposed to IR or certain alkylating agents (18). To determine whether c-Abl is involved in the regulation of SHPTP1, mouse fibroblasts deficient in c-Abl ($Abl^{-/-}$; derived from mice with targeted c-Abl disruption) (24) were assayed for SHPTP1 phosphorylation. In the $Abl^{-/-}$ cells, there was no detectable increase in tyrosine phosphorylation of SHPTP1 following exposure to IR (Fig. 1*D*). In contrast, reconstituted $Abl^{-/-}$ cells that stably express c-Abl (designated $Abl^{+}$) were found to respond to IR with increases in tyrosine phosphorylation of SHPTP1 (Fig. 1*D*). Taken together, these results provide evidence that the response to IR includes c-Abl-dependent tyrosine phosphorylation of SHPTP1.

The association between c-Abl and SHPTP1 was further analyzed by incubating U-937 cell lysates with a GST fusion protein prepared from the c-Abl SH3 domain. Adsorbates obtained with GST-Abl SH3, but not with GST, revealed binding of SHPTP1 (Fig. 2*A*). There was also no detectable binding of SHPTP1 to the C-terminal SH3 domain of the Grb2 adaptor protein (data not shown). To determine whether the interaction between c-Abl and SHPTP1 is direct, we incubated

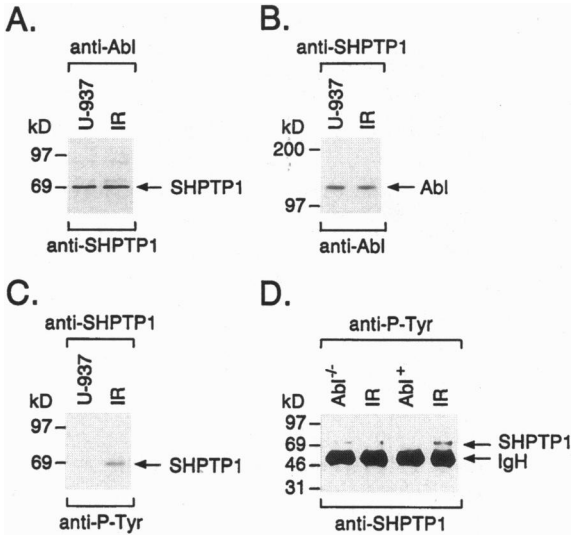


FIG. 1. *In vivo* association of SHPTP1 with c-Abl. (*A*) U-937 cells were treated with 20-Gy IR and collected 1 h later. Equal amounts of nuclear proteins were subjected to immunoprecipitation with anti-Abl. The proteins were resolved and analyzed by immunoblotting with anti-SHPTP1. (*B*) Nuclear proteins were immunoprecipitated with anti-SHPTP1 and analyzed by immunoblotting with anti-Abl. (*C*) Anti-SHPTP1 immunoprecipitates were also analyzed by immunoblotting with anti-P-Tyr. (*D*) $Abl^{-/-}$ and c-Abl reconstituted $Abl^{-/-}$ ($Abl^{+}$) cells were treated with 20-Gy IR. Equal amounts of proteins were subjected to immunoprecipitation with anti-P-Tyr and analyzed by immunoblotting with anti-SHPTP1.

beads containing glutathione-GST-Abl SH3 with purified SHPTP1 protein. The beads were washed and protein was eluted by boiling in SDS. Analysis of the eluted protein by immunoblotting with anti-SHPTP1 demonstrated a concentration-dependent interaction between the c-Abl SH3 domain and SHPTP1 (Fig. 2*B*). While these findings support direct binding, purified SHPTP1 was subjected to SDS/PAGE, transferred to nitrocellulose, and then incubated with GST-Abl SH3 or GST. Analysis of adsorbed protein with anti-GST demonstrated binding of c-Abl SH3 and not GST (Fig. 2*C*). Other studies have shown that the c-Abl SH3 domain binds to a proline-rich sequence with the consensus XPXXXXPXXP (25, 26). A potential sequence for c-Abl SH3 binding is present in SHPTP1 (amino acids 417–426; WPDHGVPSEP). To confirm binding of c-Abl to this site, we mutated prolines at positions 418 and 423 to alanine (WADHGVASEP) and asked whether this mutant interacts with GST-Abl SH3. The mutant protein is readily cleaved to a 46-kDa C-terminal fragment without SH2 domains (ΔSH2 P418A/P423A). A similar protein has been described for wild-type SHPTP1 that contains the WADHGVPSEP sequence and not the SH2 domains (ΔSH2 SHPTP1) (22). The finding that binding of the c-Abl SH3 domain to the ΔSH2 P418A/P423A mutant is decreased compared with that obtained with ΔSH2 SHPTP1 supports interaction through the WPDHGVPSEP motif (Fig. 2*D*).

SHPTP1 contains two N-terminal SH2 domains, the protein tyrosine phosphatase domain and a C terminus, which includes potential tyrosine phosphorylation sites (22). One of these sites (Y-564) is phosphorylated in T cells as a result of Lck activation (27). To determine whether and where c-Abl phosphorylates SHPTP1, we incubated anti-Abl immunoprecipitates from control and IR-treated cells with equal amounts of recombinant SHPTP1 protein (residues 1–595) (23) and [γ-$^{32}$P]ATP. Autoradiography of the reaction products demonstrated increased tyrosine phosphorylation of SHPTP1 (Fig. 3*A*). To define the sites in SHPTP1 phosphorylated by c-Abl, we used a mutant SHPTP1 (ΔC60; 1–535) which has

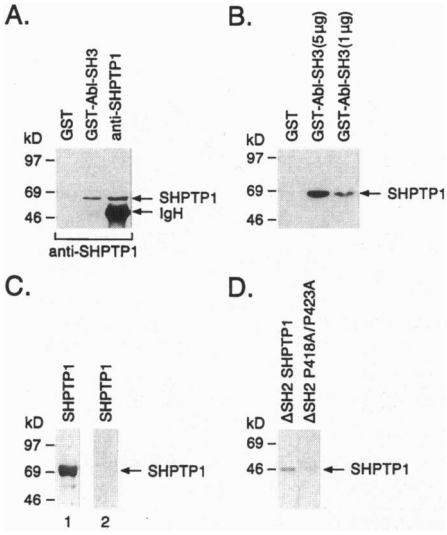


FIG. 2. SHPTP1 associates directly with the SH3 domain of c-Abl. (*A*) U-937 cell lysates were subjected to affinity chromatography with GST-Abl SH3 domain (25) or GST fusion proteins. Lysates were also subjected to immunoprecipitation with anti-SHPTP1 antibody as a positive control. Adsorbed proteins were analyzed by immunoblotting with anti-SHPTP1. (*B*) Purified SHPTP1 (23) proteins were incubated with GST or with different amounts of GST-Abl SH3 bound to glutathione-Sepharose beads. After extensive washing, proteins were boiled in the SDS buffer, resolved by SDS/PAGE, and analyzed by immunoblotting with anti-SHPTP1. (*C*) Purified SHPTP1 proteins were boiled in SDS buffer, resolved by SDS/PAGE, and transferred to nitrocellulose filters. Filters were incubated with GST or GST-Abl SH3 and analyzed by immunoblotting with anti-GST antibodies. (*D*) Purified ΔSH2 SHPTP1 or the ΔSH2 P418A/P423A mutant were incubated with GST-Abl SH3 bound to glutathione-Sepharose. The beads were washed and bound protein was eluted by boiling in SDS buffer. Eluted protein was analyzed by immunoblotting with anti-SHPTP1.

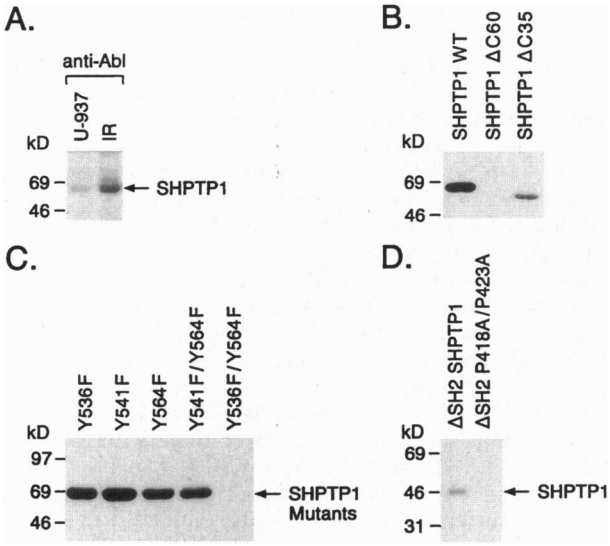


FIG. 3. Phosphorylation of SHPTP1 by c-Abl. (*A*) U-937 cells were treated with 20-Gy IR and collected after 1 h. Nuclear lysates from control and treated cells were subjected to immunoprecipitation with anti-Abl. Immune complex kinase assays were performed by incubating the resulting immunoprecipitates in kinase buffer with 4 μg of purified SHPTP1 protein and 2–5 μCi of [γ-$^{32}$P]ATP for 30 min at 30°C. (*B*) Purified truncated ΔC60 (1–535) and ΔC35 (1–560) SHPTP1 proteins (4 μg each) (23) were incubated in kinase buffer with purified c-Abl kinase for 15 min at 30°C. (*C*) Purified mutant Y536F, Y541F, Y564F, Y541F/Y564F, and Y536F/Y564F SHPTP1 proteins (4 μg each) (27) were incubated in kinase buffer with purified c-Abl kinase for 15 min at 30°C. (*D*) Purified ΔSH2 SHPTP1 and the ΔSH2 P418A/P423A mutant (4 μg each) were incubated in kinase buffer with purified c-Abl kinase for 15 min at 30°C. The reaction products were analyzed by SDS/PAGE and autoradiography.

been truncated of the last 60 amino acids (23). While this mutant failed to serve as a substrate, a ΔC35 mutant (1–560) (23) was phosphorylated by c-Abl (Fig. 3*B*), allowing focus on the potential tyrosine phosphorylation sites in the C-terminal region of SHPTP1 (Y536, Y541, and Y564). Mutation of Y536 to F failed to block phosphorylation (Fig. 3*C*). Similar results were obtained with the Y541F, Y564F, and Y541F/Y564F mutants. In contrast, there was no detectable phosphorylation of the Y536F/Y564F double mutant (Fig. 3*C*). These findings establish that c-Abl phosphorylates both the Y536 and Y564 sites. We also incubated purified c-Abl with ΔSH2 SHPTP1 and the ΔSH2 P418A/P423A mutant. The finding that c-Abl phosphorylates wild-type SHPTP1 more efficiently than the mutant supports involvement of c-Abl binding to the WPDH-GVPSEP site before the phosphorylation reaction (Fig. 3*D*).

The present findings thus provide evidence that SHPTP1 is a substrate for c-Abl. Because c-Abl is activated by certain DNA-damaging agents and contributes to activation of SAPK (18), we asked whether SHPTP1 is involved in regulation of this stress pathway. To address this issue, we transfected NIH 3T3 cells with pEBG-SAPK (17) and, after IR exposure, assayed glutathione-Sepharose protein precipitates for phosphorylation of Jun (Fig. 4*A*). Cotransfection of pEBG-SAPK and wild-type SHPTP1 blocked IR-induced activation of SAPK (Fig. 4*A*). In contrast, IR-induced SAPK activity was stimulated by cotransfection of a dominant negative SHPTP1, which expresses a C453S mutant (23) (Fig. 4*A*).

Few insights are available regarding the functional role of c-Abl. Interaction of the c-Abl SH1 domain with the retinoblastoma protein (Rb) has been reported to inhibit c-Abl kinase activity (28), while the c-Abl SH2 domain has been found to mediate binding to RNA polymerase II (29). The carboxy terminal domain of c-Abl interacts with the Crk family

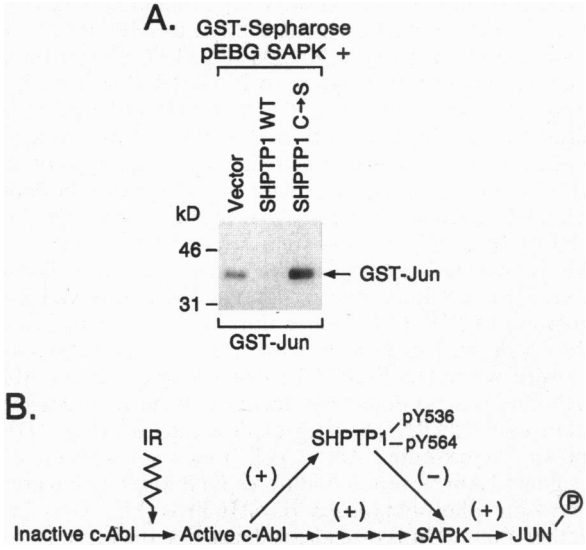


FIG. 4. Role of SHPTP1 on regulation of SAPK induced by IR. (*A*) NIH 3T3 cells were transiently cotransfected with pEBG-SAPK and pCDNA3, wild-type SHPTP1, or a phosphatase dead mutant of SHPTP1 (SHPTP1 C453S) (23). After incubation for 24 h, cells were treated with 20-Gy IR, and the cell lysates were incubated with glutathione-Sepharose. Immune complex kinase assays were performed with the resulting adsorbates using GST-Jun as a substrate. The proteins were then resolved on SDS/PAGE and analyzed by autoradiography. (*B*) Schema of potential interactions among c-Abl, SHPTP1, and SAPK.

of SH3-containing adaptor proteins (25, 26). Other studies have demonstrated that Abl-interactor proteins 1 and 2 constitutively bind to the SH3 and carboxy-terminal domains of c-Abl and suppress its transforming activity (30, 31). The present finding that the SH3 domain of c-Abl also binds constitutively to SHPTP1 supports the existence of distinct c-Abl pools. The present results also indicate that c-Abl phosphorylates SHPTP1 *in vivo* in an IR-dependent fashion. Moreover, the finding that SHPTP1 downregulates IR-induced SAPK activation further supports a role for SHPTP1 as a negative regulator of the c-Abl→SAPK cascade in the response to genotoxic stress (Fig. 4*B*). In this context, other studies have recently demonstrated a role for activated and SH-3-deleted forms of Abl in the induction of SAPK activity (32–34).

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