PHOSPHORYLATION OF DNA TOPOISOMERASE I BY THE c-Abl
TYROSINE KINASE CONFERS CAMPTOTECIN SENSITIVITY

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Abstract

DNA topoisomerase I (topoI) is involved in the regulation of DNA supercoiling, gene transcription, recombination and DNA repair. The anti-cancer agent camptothecin (CPT) specifically targets topoI. The mechanisms responsible for the regulation of topoI in cells, however, are not known. The present studies demonstrate that c-Abl dependent phosphorylation up regulates topoI activity. The c-Abl SH3 domain binds directly to the N-terminal region of topoI. The results demonstrate that c-Abl phosphorylates topoI on Y268 in core subdomain II. c-Abl-mediated phosphorylation of topoI Y268 in vitro and in cells confers activation of the topoI isomerase function. Moreover, activation of c-Abl by treatment of cells with ionizing radiation is associated with c-Abl-dependent phosphorylation of topoI and induction of topoI activity. The functional significance of the c-Abl-topoI interaction is supported by the findings that i) a topoI(Y268-F) mutant exhibits loss of c-Abl-induced topoI activity and ii) c-Abl^-/- cells are deficient in the accumulation of protein-linked DNA breaks. In addition, loss of topoI phosphorylation in c-Abl-deficient cells confers resistance to CPT-induced apoptosis. These findings collectively support a model in which c-Abl-mediated phosphorylation of topoI is functionally important to topoI activity and sensitivity to topoI poisons.
Introduction

Eukaryotic type I topoisomerases (topoI) function in the relaxation of negatively and positively supercoiled DNA (1-4). TopoI-mediated relaxation is accomplished by the introduction of a single-stranded nick in the phosphodiester backbone, rotation of the complementary DNA strand through the break and relief of torsional strain. Catalysis of the single-stranded nick involves the formation of a covalent bond between the 3'‐end of the DNA strand break and the active site tyrosine in the carboxy‐terminal region of topoI (5,6). Following relaxation, reversal of the transesterification reaction results in religation of the phosphodiester bond and release of topoI. The reduction of torsional stress is essential to DNA replication (1,4). TopoI has also been shown to participate in RNA polymerase II‐mediated transcription (7,8), in DNA repair (9‐11) and in RNA splicing (12‐14). Despite the importance of topoI to cellular functions, little is known about the mechanisms responsible for the regulation of this enzyme.

Human topoI is a nuclear protein with 765 residues, a predicted molecular mass of 91 kDa and four domains. The N‐terminal non‐conserved domain from Met1 to Lys197 contains four putative nuclear localization signals, is sensitive to proteolysis and can be deleted without effect on topoI activity (15‐17). The conserved core domain extends from Glu198 to Ile651 and is followed by a short linker domain from Asp652 to Glu696. The highly conserved C‐terminal domain extends from Gln697 to Phe765, contains the active site tyrosine at position 723 and is required
for topoI activity (6). Crystal structures of the core and C-terminal domains in covalent and non-covalent complexes with duplex DNA have shown that the enzyme clamps around B-form DNA (18). Moreover, the linker region assumes a coiled-coil configuration and protrudes away from the rest of the protein (19).

TopoI is a cellular target for the plant alkaloid camptothecin (CPT) (3,20-22). The available evidence supports a model in which CPT binds to the topoI-DNA complex subsequent to DNA cleavage and covalent attachment of topoI to DNA (23,24). CPT then stabilizes the cleavage complexes by inhibiting topoI-mediated DNA religation (25,26). A CPT binding mode has been proposed in which CPT interacts with both DNA strands and topoI residues Arg364, Asp533 and Asn722 (18). Stabilization of the cleavage complexes by CPT is believed to induce lethality as a result of collisions with proteins involved in DNA replication and transcription (25-28). In addition, CPT-induced stabilization of the topoI-DNA complex is associated with conversion of single strand nicks to irreversible double strand breaks (21,29-32). Thus, cells selected for resistance to CPT express decreased levels of topoI (33-35) or certain point mutations in the TOPOI gene (36). Posttranslational modification of topoI has been shown to regulate topoI activity. Protein kinase C-dependent phosphorylation up-regulates topoI activity and thereby increases CPT sensitivity (37).

Activity of the ubiquitously expressed c-Abl protein tyrosine kinase is tightly regulated in cells (38,39). Nuclear c-Abl
associates with the DNA-dependent protein kinase (DNA-PK) complex (40,41) and with the product of the gene mutated in ataxia telangiectasia (ATM) (42,43). The DNA-PK catalytic subunit and ATM are members of a family of phosphatidylinositol (PI) 3-kinase-like enzymes involved in regulation of the cell cycle, recombination, control of telomere length and the DNA damage response (44). Importantly, c-Abl is activated by DNA-PK and ATM in cells exposed to ionizing radiation (IR) and other genotoxic agents (40,42,43,45,46). The available evidence indicates that activation of nuclear c-Abl contributes to DNA damage-induced growth arrest and apoptosis by mechanisms in part dependent on p53 and its homolog p73 (47-51). Other studies have demonstrated that nuclear c-Abl interacts with the human catalytic subunit of telomerase (hTERT) and thereby regulates telomere length (52). These findings have supported a role for nuclear c-Abl in converting DNA damage into signals that control the genotoxic stress response.

Because c-Abl is activated by DNA strand breaks (45) and topoI is involved in the generation of these lesions (35), we investigated whether c-Abl interacts with topoI. The results demonstrate that c-Abl phosphorylates topoI and that c-Abl functions in the induction of topoI activity. We also show that c-Abl-mediated activation of topoI is important at least in part, in imparting cellular sensitivity to CPT.
Materials and Methods

**Cell culture.** Human 293 kidney cells, MCF-7 breast carcinoma cells expressing neo or c-Abl(K-R) (53), wild-type mouse embryo fibroblasts (MEFs) c-Abl−/− MEFs (54) and c-Abl−/− reconstituted to stably express c-Abl (designated c-Abl+) (45) were grown in Dulbecco’s Modified Eagle’s Medium containing 10% heat-inactivated fetal bovine serum (HI-FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Human U-937 myeloid leukemia cells were grown in RPMI 1640 medium supplemented with 10% HI-FBS, antibiotics and L-glutamine. Cells were treated with 10 µM CPT (Sigma). Irradiation was performed at room temperature using a Gammacell-1000 (Atomic Energy of Canada, Ottawa, Ontario, Canada) under aerobic conditions with a 137Cs source emitting at a fixed dose rate of 0.76 Gy/min as determined by dosimetry.

**Immunoprecipitation and immunoblot analysis.** Cell lysates were prepared for immunoprecipitation as described (45). Soluble proteins were incubated with anti-c-Abl (Santa Cruz) or anti-topoI (Topogen, Inc.) as described (45). The immunoprecipitates were subjected to immunoblotting with anti-c-Abl or anti-topoI. Antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL detection system, Amersham Biosciences, Piscataway, NJ).

**Fusion protein binding assays.** GST and GST-c-Abl SH3 were purified by affinity chromatography using glutathione-sepharose beads and equilibrated in lysis buffer. Cell lysates were incubated with 2µg of immobilized GST or GST-Abl SH3 for 2 hours
at 4°C. GST-Grb2-SH3 fusion protein was used as an extra control. The resulting protein complex were washed with lysis buffer and analyzed by immunoblot analysis with anti-topoI. In the reciprocal experiment GST-topoI or fragments of topoI linked to GST were separately incubated with lysates and the adsorbates were analyzed by immunoblot analysis with anti-c-Abl.

**Direct interaction of c-Abl with topoI.** Purified GST-c-Abl, bound to glutathione sepharose beads (approximately 10 µg) was incubated with 5 units of thrombin (Amersham Biosciences, Piscataway, NJ) for four hours at 25°C. 5 µg of c-Abl was incubated with 4 µg of GST-topoI bound to glutathione sepharose beads at 4°C for two hours. The beads were washed extensively with PBS and the adsorbates were eluted with high (500 mM NaCl). The eluate was analyzed by SDS-PAGE followed by silver staining. The protein bands were excised and processed for trypsin digestion. The resulting peptides were analyzed by MALDI-TOF-MS (Voyager DE-PRO, ABI, Framingham, MA) and electrospray ionization mass spectrometry (ESI-MS). ESI-MS and MS/MS were performed using an electrospray iontrap, LCQ-DECA (Thermo Electron, CA). The tryptic peptides were fractionated on capillary HPLC C-18 column coupled with mass spectrometer. Tandem mass spectra were acquired using Ar as the collision gas and sufficient collision energy to obtain complete sequence information of the precursor ion. MS and MS/MS data was then analyzed by BioWorks 3.0.
software package (Thermo Electron, CA). In other experiment c-Abl was incubated with GST-topoI and adsorbates were analyzed by immunoblotting with anti-c-Abl.

**In vitro phosphorylation studies.** Purified GST-topoI (55) was incubated with c-Abl, c-Abl(K-R) in kinase buffer (20 mMTris-HCl, pH 7.4, 10 mM MgCl₂ and 10 mM MnCl₂) containing [γ-³²P]ATP or cold ATP for 30 min at 30°C. Kinase-active c-Abl and kinase-inactive c-Abl(K-R) were purified from baculovirus-infected insect cells (56). The c-Abl proteins were purified by binding to glutathione beads and washing. Analysis of the c-Abl preparations by SDS-PAGE and protein staining demonstrated >90% purity. In certain experiments, the c-Abl phosphorylated GST-topoI was incubated with purified SHPTP1 protein tyrosine phosphatase (57) for 5 min at 30°C. The reaction products were analyzed i) by SDS-PAGE and autoradiography, ii) in assays of topoI activity, and iii) in mapping the phosphorylation site.

**Preparation of nuclear lysates.** Nuclear lysates were prepared as described (36,58). Protein concentration was determined by the Bio-Rad protein assay.

**TopoI activity assays.** TopoI activity was measured by DNA relaxation assays as described (36) in a reaction buffer containing 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol. The gels were imaged by
Identification of in vitro tyrosine phosphorylation sites. Purified GST-topoI was incubated with c-Abl and \([γ-^{32}P]\)ATP. The reaction products were subjected to SDS-PAGE. The topoI band was identified by Coomassie blue staining and excised from the gel. In gel digestion with trypsin was performed as described (60,61). In brief, corresponding slice of gel was cut into small pieces and dehydrated with acetonitrile. The content was rehydrated with 10mM DTT in 100mM ammonium bicarbonate and incubated at 56°C for one hour. Following dehydration with acetonitrile, gel pieces were suspended in trypsin(12.5 ng/µl) in 50 mM ammonium bicarbonate. In gel digestion was carried out at 37°C for 10-12 hours. The peptides were extracted in 50% acetonitrile / 5% formic acid.

For \(^{32}P\)-labeled topoI, the trypsin-digested peptides were fractionated by RP-HPLC. Aliquots of the fractions were assayed for \(^{32}P\). Positive fractions were subjected to Edman sequencing and a part of the digest was directly analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using a Voyager DE-PRO (ABI, Framingham, MA). The Edman sequencing was performed for fourteen cycles and the elute of every cycle was assayed for \(^{32}P\).
Identification of in vivo tyrosine phosphorylation sites. Nuclei isolated in NB buffer were resuspended in lysis buffer (50 mM NaH₂PO₄, pH 8.0, 500 mM NaCl, 10 mM imidazole, 10 mM NaF, 1 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 2 mM PMSF). The suspension was gently rotated for 1 h at 4°C and then centrifuged at 17,000xg for 10 min. The supernatant was incubated with pre-equilibrated Ni-NTA matrix (Quagen, Valencia, CA). The matrix was washed with lysis buffer containing 25 mM imidazole. Proteins were eluted with lysis buffer containing 100 mM imidazole, concentrated and separated by SDS-PAGE. The topoI band was identified by staining, excised and subjected to in gel trypsin digestion. Masses of the trypsin-digested peptides were analyzed by MALDI-TOF-MS.

Site-directed mutagenesis. Tyrosine 268 in topoI was mutated to phenylalanine using the site-directed mutagenesis kit (Stratagene).

Analysis of PLDB and DNA fragmentation. TopoI protein-linked DNA breaks (PLDB) were assayed as described (62). Quantification of DNA fragmentation by alkaline elution assays was performed as described (63).

Analysis of sub-G1 DNA content. Cells were fixed with ethanol and stained with propidium iodide. DNA content was assessed by FACScan (Becton-Dickinson).
**Results**

**c-Abl associates with topoI.** To determine whether c-Abl forms a complex with topoI, cell lysates were subjected to immunoprecipitation with anti-c-Abl. Analysis of the immunoprecipitates with anti-topoI demonstrated the coprecipitation of c-Abl and topoI (Fig. 1A). In the reciprocal experiment, immunoblot analysis of anti-topoI immunoprecipitates with anti-c-Abl confirmed the association of c-Abl and topoI in cells (Fig. 1B). Based on the total amounts of topoI and c-Abl in the lysates subjected to immunoprecipitation, approximately 5% of the c-Abl pool and 4% of the topoI pool contributes to the formation of the c-Abl-topoI complexes. To further assess the interaction between c-Abl and topoI, cell lysates were incubated with GST, GST-Grb2-SH3 or GST-c-Abl-SH3. Analysis of adsorbates to glutathione beads by immunoblotting with anti-topoI demonstrated binding of topoI to GST-c-Abl-SH3, but not GST or GST-Grb2-SH3 (Fig. 1C). In other experiments, fragments of topoI linked to GST were incubated with cell lysates. Immunoblot analysis of the adsorbates with anti-c-Abl demonstrated no detectable binding with topoI deleted at amino acids 1-210 (Fig. 1D). Localization of the c-Abl binding site to the N-terminal region of topoI was confirmed by the demonstration that c-Abl binds to GST-topoI(1-250) (Fig. 1D). To assess direct interaction, purified c-Abl recombinant protein was incubated with GST-topoI linked to beads. After extensive washing the adsorbates were analyzed by immunoblotting with anti-c-Abl. The results demonstrate a direct interaction of c-Abl with topoI (Fig 1E).
These results demonstrate that c-Abl binds directly to topoI and that the c-Abl SH3 domain associates with the N-terminal region of topoI.

**Topo I associates directly with c-Abl.** To determine the direct interaction, purified c-Abl was incubated with GST-topoI linked to beads. After extensive washing with PBS the adsorbates were analyzed by SDS-PAGE and silver staining. Two topoI interacting proteins, 140 kDa and 100 kDa, were visualized on SDS-PAGE (fig 2A). Both proteins were trypsin digested and the resulting peptides were analyzed by MALDI-TOF-MS and ESI-MS/MS. The MALDI-TOF-MS generated spectrum (data not shown) was subjected to database search using Protein Prospector. The protein was identified as Proto-oncogene tyrosine-protein kinase ABL1 (p150) (c-Abl); NCBI accession # 125135 and swissprot accession # P00519. A fraction of tryptic peptides were also analyzed by electrospray iontrap tandem mass spectrometry. The peptides were separated in C-18 capillary column and total ion chromatogram was recorded (fig 2B, upper panel). MS and MS/MS data were generated and analyzed using BioWorks 3.0 software package to identify the protein. Based on the peptide sequence data (MS/MS) the protein was identified as c-Abl. Four peptides with 1.5 or higher Xc value were identified as c-Abl peptide sequences. A peptide with m/z 644.17 2+ was identified as c-Abl peptide eluted between 55-56 min.
(represented by shaded area, fig. 2B upper panel) and MS scan representing the peptide (fig 2B middle panel). The MS/MS data analysis of peptide 644.17 2+ (fig 2B lower panel) identified c-Abl amino acid ‘EAINKLESNL’. The y and b ion coverage of peptide fragments provided xC value of 2.23. The other peptide sequences representing c-Abl were: LKPAPPPPPACTGK (xC-3.02); LLSSVKEISDIVRR (xC-2.97) and IASGTITK (xC-1.9). Four peptide sequences with high xC value representing y and b ion series, representing c-Abl kinase clearly demonstrate that the full length c-Abl (140kDa) and a fragment of c-Abl (100 kDa) binds topoI directly. The identification of c-Abl was further supported by MALDI-TOF-MS analysis and mass fingerprinting.

c-Abl phosphorylates and activates topoI in vitro. To determine whether c-Abl phosphorylates topoI, purified c-Abl was incubated with GST-topoI in the presence of $[\gamma^{32P}]$ATP. GST and the c-Abl substrate, GST-Crk(120-225) (64), were included in similar reactions as controls. Analysis of the reaction products by SDS-PAGE and autoradiography demonstrated that, like Crk(120-225), topoI is subject to c-Abl phosphorylation (Fig. 3A). As shown previously (45), GST was not a substrate for the c-Abl kinase (data not shown). To quantitate the c-Abl mediated topoI phosphorylation, we next estimated the molar incorporation of ATP. Purified c-Abl was incubated with GST-topoI in the presence of $[\gamma^{32P}]$ATP and the reaction product was analyzed by SDS-PAGE and
coomassie staining. The samples in duplicate were analyzed by SDS-PAGE and autoradiography (fig 3A lower). Coomassie stained GST-topoI and GST-Crk proteins were cut from the gel and assayed for $^{32}$P. The results indicated that 2 pmol c-Abl can incorporate 10.6 pmol of ATP on 100 pmol GST-topoI. GST-Crk 120-225 recorded four fold more incorporation of ATP. To assess the effects of c-Abl phosphorylation on topoI activity, assays were performed that measure the effects of topoI on relaxation of supercoiled DNA. In these experiments, GST-topoI was preincubated with c-Abl or kinase-inactive c-Abl(K-R) in the presence of ATP. The topoI was then assayed at increasing concentrations for DNA relaxation activity. The results demonstrate that phosphorylation of topoI by c-Abl is associated with increased activity in converting supercoiled DNA to the relaxed form (Fig. 3B, left). As determined by image analysis of the activity gel, 2.7 ng of c-Abl phosphorylated topoI relaxed nearly 90% of the supercoiled DNA, while 2.5 ng of control topoI or topoI incubated with c-Abl(K-R) was associated with 20% relaxation (Fig. 3B, middle). Moreover, 4 ng of c-Abl phosphorylated topoI conferred complete relaxation, while unphosphorylated topoI relaxed 40-50% of the supercoiled DNA (Fig. 3B, middle). Similar findings were obtained in four independent experiments (Fig. 3B, right). To confirm that tyrosine phosphorylation of topoI is responsible for activation of the isomerase function, topoI was incubated with c-Abl and [$\gamma$-$^{32}$P]ATP. Treatment of the $^{32}$P-labeled topoI with the SHPTP1 protein tyrosine phosphatase was associated with a decrease in tyrosine phosphorylation (Fig. 3C, left). Similar studies were
performed with topoI that had been preincubated with c-Abl and cold ATP to assess the effects of SHPTP1 on topoI activity. The results demonstrate that SHPTP1 attenuates c-Abl-mediated activation of topoI (Fig. 3C, middle and right). These findings collectively demonstrate that c-Abl phosphorylates and activates topoI in vitro.

**c-Abl-dependent phosphorylation and activation of topoI in vivo.** To determine whether topoI is phosphorylated by a c-Abl-dependent mechanism in cells, lysates from human MCF-7 breast carcinoma cells stably expressing the empty neo vector or the kinase-inactive, dominant-negative c-Abl(K-R) were subjected to immunoprecipitation with anti-topoI. Analysis of the immunoprecipitates with anti-P-Tyr demonstrated that tyrosine phosphorylation of topoI is decreased in MCF-7/c-Abl(K-R), as compared to MCF-7/neo, cells (Fig. 4A). Reprobing the membrane with anti-topoI demonstrated equal amounts of topoI protein (Fig. 4A). Immunoprecipitation of the lysates with anti-P-Tyr and immunoblot analysis of the supernatants demonstrated a 25% decrease in the amount of topoI. These findings indicate that about 1 of 4 topoI molecules is subject to tyrosine phosphorylation and/or that topoI coprecipitates with other tyrosine phosphorylated proteins. To assess topoI activity, nuclear extracts were assayed for relaxation of supercoiled DNA. The results show that topoI activity is decreased in MCF-7/c-Abl(K-R), as compared to MCF-7/neo cells (Fig. 4B). Image analysis of the activity gel demonstrated that 4 µg of nuclear
extract from MCF-7/neo cells relaxed 70% of the supercoiled DNA, while the same amount of nuclear lysate from MCF-7/neo cells conferred 40% relaxation (Fig. 4B). To further assess involvement of c-Abl in the regulation of topoI, studies were performed on wild-type (c-Abl+/+) and c-Abl-/- MEFs (54). Immunoblot analysis of anti-topoI immunoprecipitates with anti-P-Tyr demonstrated that tyrosine phosphorylation of topoI from the c-Abl-/- cells is decreased compared to that found in wild-type cells (Fig. 4C, left). Reprobing the blots with anti-topoI demonstrated equal amounts of topoI in both cell types (Fig. 4C, left). Of note, expression of topoI proteins in MEFs differs from that in MCF-7 cells, particularly in terms of the isomerase-active 68 kDa form (16,65,66). Densitometric analysis of the anti-P-Tyr signals from 4 separate experiments demonstrated that tyrosine phosphorylation of topoI is decreased by about 80% in c-Abl-/- cells (Fig. 4C, right). Analysis of nuclear extracts for relaxation of supercoiled DNA showed that topoI activity is decreased in c-Abl-/-, as compared to wild-type, cells (Fig. 4D). Image analysis of the activity gels from 4 separate experiments demonstrated that 9 µg of nuclear extract from c-Abl-/- cells relaxed about 40% of the supercoiled DNA, while that amount of nuclear lysate from c-Abl+/+ cells was associated with 90% relaxation (Fig. 4D). These findings demonstrate that c-Abl phosphorylates topoI in vivo and c-Abl dependent phosphorylation upregulates topoI activity.

c-Abl-dependent phosphorylation and activation of topoI in response to genotoxic stress. Exposure of cells to
IR and other genotoxic agents is associated with activation of the c-Abl kinase function (45). To assess the effects of IR on the interaction of c-Abl and topoI, lysates from irradiated wild-type and c-Abl\(^{-/-}\) cells were subjected to immunoprecipitation with anti-topoI. Immunoblot analysis of the immunoprecipitates with anti-P-Tyr demonstrated an IR-dependent increase in tyrosine phosphorylation of topoI in wild-type cells (Fig. 6A). By contrast, IR had little effect on tyrosine phosphorylation of topoI in c-Abl\(^{-/-}\) cells (Fig. 6A). These findings indicate that IR induces tyrosine phosphorylation of topoI by a c-Abl-dependent mechanism. Analysis of topoI activity indicated that IR treatment of wild-type cells is associated with increased relaxation of supercoiled DNA (Fig. 6B). By contrast, IR had less of an effect on topoI activity in c-Abl\(^{-/-}\) cells (Fig. 6B). Image analysis of the gel confirmed that IR induces topoI activity by a c-Abl-dependent mechanism (Fig. 6C). Of note, the change in tyrosine phosphorylation of topoI in response to IR remained more pronounced in comparison with the basal tyrosine phosphorylation. With higher basal tyrosine phosphorylation (fig.4C) the immunoprecipitation technique does not allow to see the difference as only 1-2% of cellular topoI is immunoprecipitated.

**Identification of the c-Abl-mediated topoI tyrosine phosphorylation site.** To identify the in vitro phosphorylation site purified GST-topoI was incubated with c-Abl and [\(\gamma^{32}\text{P}\)]ATP. The reaction products were subjected to SDS-PAGE. The topoI band was identified by Coomassie blue staining and excised from the
gel. In gel digestion with trypsin was performed and the peptides were fractionated by RP-HPLC on a cation exchange column. The fractions were assayed for $^{32}$P. Out of 60 fractions 9 showed significantly higher levels of $^{32}$P. All nine fractions were analyzed by MALDI-TOF-MS and fraction 26 (fig.5A) was further analyzed by Edmon sequencing. The 263-275 amino acid region of topoI was sequenced (fig 5B). Further analyses of sequence elute showed higher activity of sixth sequence cycle representing topoI Y268 (fig 5B).

To define the topoI sites phosphorylated by c-Abl in wild-type MEFs, topoI was purified by binding of the histidine-rich N-terminal region to Ni-conjugated beads. The purified topoI was subjected to SDS-PAGE, excised and then analyzed by MALDI-TOF-MS. Y268 was identified as phosphorylated analyzed by MALDI-TOF-MS and Edman sequencing (data not shown). To confirm these findings, c-Abl was incubated with wild-type topoI or a Y268F mutant in the presence of $[\gamma^{32}P]$ATP. Analysis of the reaction products demonstrated that c-Abl phosphorylation is decreased as a result of the Y268F mutation (Fig. 7A). Analysis in DNA relaxation assays demonstrated that the topoI(Y268F) mutant exhibits less isomerase activity as compared to wild-type topoI (Fig. 7B). In addition and unlike wild-type topoI (Fig. 3B), incubation of topoI(Y268F) with c-Abl and ATP had no detectable effect on DNA relaxation activity (Fig. 7C). These findings demonstrate that topoI Y268 is phosphorylated by c-Abl and that this site is functionally important for topoI activity.
Functional significance of the interaction between c-Abl and topoI Y268. As there is less topoI activity and no detectable phosphorylation of topoI Y268 in c-Abl−/− MEFs, the response of these cells to CPT was compared to that of wild-type cells. CPT induces protein-linked DNA breaks (PLDB) that are stabilized intermediates of topoI activity (62). Using an in vivo KCl-SDS coprecipitation assay, analysis of PLDB demonstrated a CPT concentration-dependent increase in wild-type MEFs (Fig. 8A). By contrast, the induction of PLDB was attenuated in the c-Abl−/− cells (Fig. 8A). To extend the analysis of c-Abl−/− cells, CPT-induced DNA fragmentation was measured by quantitating the formation of DNA single-strand breaks (SSB) in alkaline elution assays. The results demonstrate that CPT treatment of c-Abl−/− cells is associated with decreased formation of DNA SSB as compared to that found in c-Abl+/+ cells (Fig. 8B). Moreover, the finding that CPT treatment of c-Abl+ cells is associated with formation of DNA SSB at a level similar to that in c-Abl+/+ cells provided support for the involvement of c-Abl in the response (Fig. 8B). To determine whether decreased formation of PLDB DNA SSB affects sensitivity to CPT-induced apoptosis, cells were analyzed for sub-G1 DNA content. The results demonstrate that the c-Abl−/− cells are significantly less sensitive to the apoptotic effects of CPT (Fig. 8C). Thus, 25% of the CPT-treated c-Abl+/+ cells contained sub-G1 DNA as compared to only 0.5% for c-Abl−/− cells exposed to this agent (Fig. 8C). The finding that c-Abl+ cells respond to CPT with an apoptotic response similar to that found for c-Abl+/+ cells provided further support for a c-Abl-
dependent mechanism (Fig. 8C). These findings collectively demonstrate that the c-Abl dependent phosphorylation of topoI is of functional importance to inhibition of topoI activity by CPT.
Interaction between topoI and nuclear c-Abl.

Proliferating mammalian cells require topoI for viability (1,3). However, the functions of topoI that are indispensable for growth are not yet known. Moreover, little is known about the interactions between topoI and other nuclear proteins, particularly the significance of such interactions to topoI function and the sensitivity of this enzyme to CPT. TopoI is associated with actively transcribed regions of chromatin (68) and functions as a coactivator of transcription (7,8). Furthermore, topoI associates with the TATA-binding protein (7) and enhances TFIID-TFIIA complex assembly during activation of RNA polymerase II-mediated transcription (69). The physical interaction between topoI and nucleolin has also underscored a potential role for topoI in RNA polymerase I-mediated transcription (55). In addition, binding of topoI to nucleolin is involved in the cellular localization of topoI (70). Other studies have shown that human topoI interacts with a RING finger/arginine-serine protein named topors (71) and with the SF2/ARF RNA splicing factor (12-14).

The present studies demonstrate that topoI associates with the nuclear c-Abl kinase. The results show that the c-Abl SH3 domain interacts with the N-terminal 1-250 amino acids of topoI. The c-Abl SH3 domain binds to a proline-rich sequence with the consensus XPXXXXPXXP (72,73). A potential site for c-Abl SH3 binding is located at amino acids 225-233 (PVFAPPYEP) in the conserved topoI core domain. The results also indicate that
deletion of amino acids 1-210 interferes with c-Abl SH3 binding to topoI, perhaps by altering conformation of the proline-rich motif. Nucleolin (55), SF2/ASF (14), topors (71) and SV-40 T antigen (71,74) also bind to topoI within the N-terminal 210 amino acids which are dispensable for topoI activity. Subdomains I and II of the topoI core form the top half or "cap" of the enzyme, bind to DNA and are essential for the isomerase function (18). c-Abl also binds to DNA, prefers sequences containing an AAC motif and exhibits a higher affinity for bent strands (75,76). Our in vitro binding studies, however, demonstrate that the interaction between topoI and c-Abl is not dependent on the presence of DNA. Direct binding experiments with purified c-Abl and GST-topoI demonstrate that the interaction is not mediated by DNA or any other protein (fig. 1E,2A and B). In contrast to the findings with c-Abl, we have not detected complexes of topoI and c-Abl-related Arg kinase in cells. One potential explanation is that Arg is expressed predominantly in the cytosol and thereby would exhibit little interaction with nuclear topoI.

**c-Abl phosphorylates and activates topoI.** The present results further demonstrate that the interaction between topoI and c-Abl involves c-Abl-mediated phosphorylation of topoI. In vitro and in vivo studies have identified topoI Y268 as a c-Abl phosphorylation site. TopoI Y268 is located in the α2 helix of subdomain II, which extends away from the body of the molecule as it wraps around the DNA duplex (18). As such, the c-Abl phosphorylation site is present on the surface of topoI in a
complex with DNA. By contrast, sites of mutations in topoI that confer resistance to CPT have been localized to areas of the core subdomains and the C-terminal domain that interact with DNA (18). These findings support a model in which topoI and c-Abl can interact when topoI is wrapped around the DNA duplex or when unbound to DNA. In this context, in vitro studies have demonstrated that c-Abl binds to and phosphorylates topoI in the absence or presence of DNA (data not shown).

The functional significance of c-Abl-mediated topoI phosphorylation is supported by the finding that this event is associated with stimulation of topoI activity. In vitro studies show that topoI which has been phosphorylated by c-Abl is more active in DNA relaxation assays. Moreover, studies in i) MCF-7 and MCF-7/c-Abl(K-R) cells and ii) wild-type and c-Abl-/- MEFs demonstrate that topoI is phosphorylated on tyrosine by a c-Abl-dependent mechanism and that topoI activity is decreased in the absence of c-Abl activity. Of note, the demonstration that, as compared to controls, tyrosine phosphorylation of topoI is decreased by approximately 80% in c-Abl-/- MEFs and MCF-7/c-Abl(K-R) cells supports phosphorylation of topoI in part by other tyrosine kinases. The findings also support a role for the Y268 site in the regulation of topoI activity. Mutation of the Y268 site was associated with the inhibition but not complete abrogation of c-Abl-mediated tyrosine phosphorylation in vitro and activation of topoI. This findings clearly indicate the possibility of multiple c-Abl dependent phosphorylation sites in topoI. Taken together, These results demonstrate that topoI is
subject to posttranslational modification that affects the activity of topoI.

**c-Abl-dependent activation of topoI in the DNA damage response.** The nuclear form of c-Abl is activated in the cellular response to DNA damage (45). Previous work has shown that c-Abl is activated by DNA-PK and ATM in cells exposed to IR and other genotoxic agents (40,42,43,45,46). The results of the present studies demonstrate that IR induces c-Abl-dependent tyrosine phosphorylation of topoI. Thus, IR treatment of wild-type, but not c-Abl−/−, MEFs results in phosphorylation of topoI on tyrosine. The results also demonstrate that phosphorylation of topoI by c-Abl in the IR response results in the stimulation of topoI activity. These findings support a model in which induction of c-Abl activity by DNA lesions transduces signals that confer activation of topoI.

Studies have linked topoI to the recognition of DNA lesions, including mismatched bases, abasic sites, cyclopyrimidine dimers and deaminated cytosines (77-81). Moreover, genotoxic agents, such as mitomycin C, that cause DNA strand cross-links, have been shown to induce topoI activity (82). Notably, mitomycin C-induced activation of topoI has been attributed to interactions between topoI and the p53 tumor suppressor (82-84). Other studies have demonstrated that topoI is subject to SUMO-1 modification in the response to DNA damage (85). The association between topoI and wild-type p53 occurs transiently in response to genotoxic stress, while binding of topoI to mutant p53 is constitutive (84).
also binds to p53 in the response to genotoxic stress and results in stabilization of the p53 protein by a mechanism involving Mdm2 (47,86). A proline-rich sequence in the C-terminus of c-Abl is necessary for binding of c-Abl to p53 (47,87). Other studies have demonstrated that c-Abl binds to the C-terminus of p53 (amino acids 363-393) (88), while the topoI binding site is located between amino acids 302-321 (84). Taken together, these findings and those in the present study indicate that both c-Abl and p53 contribute to the activation of topoI.

**c-Abl dependent activation and the sensitivity of topoI to CPT.** Yeast cells devoid of topoI are resistant to the lethal effects of CPT (89,90). Moreover, cells that overexpress topoI are hypersensitive to CPT and cells selected for resistance to CPT exhibit decreased levels of topoI expression (79,90-92). Other findings have demonstrated that certain mutations in topoI, particularly those involved in interactions with duplex DNA (18), confer CPT resistance. There is no available information, however, regarding the regulation of topoI activity as a factor in the sensitivity of cells to CPT. The present results demonstrate that c-Abl-mediated phosphorylation of topoI increases the isomerase activity. In concert with these observations, the results further show that c-Abl<sup>-/-</sup> cells are resistant to CPT-induced PLDB. The c-Abl<sup>-/-</sup> cells also exhibit decreased levels of topoI activity as a consequence of loss of tyrosine phosphorylation and are less sensitive to CPT-induced apoptosis. These findings collectively demonstrate that c-Abl-mediated
phosphorylation is functionally important to topoI activity and sensitivity to topoI poisons.
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Figure Legends

Fig. 1. TopoI interacts with c-Abl in cells and in vitro.
A. Lysates from non-transfected 293 cells were subjected to immunoprecipitation with anti-c-Abl or IgG. The immunoprecipitates were analyzed by immunoblotting with anti-topoI. Lysate not subjected to immunoprecipitation was used as a control. The supernatant after anti-c-Abl immunoprecipitation was subjected to immunoblot analysis with anti-topoI. The finding that approximately 95% of the topoI protein remains in the supernatant indicated that 1 of 20 topoI molecules associates with c-Abl. B. Anti-topoI immunoprecipitates obtained from 293 cell lysates were subjected to immunoblotting with anti-c-Abl. C. 293 cell lysate (800 µg) was incubated with GST, GST-Grb2-SH3 or GST-c-Abl-SH3. The adsorbates were subjected to immunoblotting with anti-topoI. Lysate (60 µg) not incubated with GST proteins was included in the immunoblot analysis as a control. D. 293 cell lysate was incubated with GST-topoI(221-765), GST-topoI(1-250) or GST-topoI(1-765) full length. The adsorbates were analyzed by immunoblotting with anti-c-Abl. E. Purified c-Abl protein was incubated with GST-topoI and adsorbates were analyzed by SDS-PAGE and immunoblot analysis with anti c-Abl.

Fig. 2. TopoI associates with c-Abl directly.
A. Purified GST-c-Abl was treated with thrombin to cleave GST. c-Abl was then incubated with GST-topoI for one hour. After extensive wash with PBS the adsorbates were analyzed by SDS-PAGE and silver staining. Thrombin cleaved c-Abl
showed multiple protein bands while no proteins were observed in GST lane. Two proteins with molecular mass of 140 kDa and 100 kDa were observed in GST-topoI lane (fig 2A). B. Two proteins visualized in SDS-PAGE (fig 2A) were excised and subjected to trypsin digestion. The tryptic peptides were analyzed by MALDI-TOF-MS and electrospray ionization tandem mass spectrometry (ESI-MS/MS). A representative total ion chromatogram from capillary liquid chromatography mass spectrometric analysis of the in gel digest is shown (fig 2A) (upper panel). Mass spectrum from LC-MS elution at time 56 to 57 min (as indicated by shaded region in upper panel), peaks are labeled with m/z value, (middle panel). Data base search identified peak with m/z 644.17 ^2+ as c-Abl peptide and the MS/MS scan was performed on the peptide to determine the amino acid sequence based on y and b ion series (fig. 2B lower panel).

**Fig. 3.** c-Abl-mediated phosphorylation of topoI induces topoI isomerase activity. A. GST-Crk(120-225) (4 µg, 100 nmoles; lanes 1, 3 and 5) or GST-topoI (12 µg, 100 nmoles; lanes 2, 4 and 6) was incubated with purified c-Abl (2 pmoles) and [γ-32P]ATP at 30°C for the indicated times. The reaction products were analyzed by SDS-PAGE and autoradiography (upper panel). 100p moles of GST-topoI was incubated with GST cleaved purified c-Abl (2 pmoles) and [γ-32P] ATP at 30°C for 30 min. The reaction products were analyzed by SDS-PAGE and autoradiography (fig 2A lower). B.
GST-topoI (5 µg) was incubated with 20 ng kinase-active c-Abl or kinase-inactive c-Abl(K-R) and ATP. The GST-topoI proteins were incubated at increasing concentrations (1.4, 2.7, 4, 5.4 and 6.7 ng) with supercoiled DNA for 30 min at 37°C. Supercoiled (SC) and relaxed (R) DNA was analyzed by agarose gel electrophoresis (left panel). The gel was subjected to image analysis. The intensities of the SC DNA bands were compared to that obtained in the absence of topoI (last lane). TopoI (0); topoI + c-Abl (n); and topoI + c-Abl(K-R) (†) (middle panel). The c-Abl and c-Abl(K-R) preparations exhibited no detectable isomerase activity (data not shown). Similar results for % SC DNA (mean ± SD) were obtained in four independent experiments each using 4 ng topoI (right panel). C. GST-topoI was incubated with c-Abl and [γ-32P]ATP. The reaction products were treated without or with SHPTP1 and then analyzed by SDS-PAGE and autoradiography (left panel). GST-topoI was incubated with c-Abl and cold ATP. The reaction products were treated without (n) or with (†) SHPTP1 and then incubated with SC DNA. The DNA was analyzed by agarose gel electrophoresis (middle panel) and the SC DNA was quantitated by image analysis (right panel).

**Fig. 4.** c-Abl phosphorylates and activates topoI in cells. A. Lysates from MCF-7 cells stably expressing the empty neo vector or c-Abl(K-R) were subjected to immunoprecipitation with anti-topoI. The immunoprecipitates were analyzed by immunoblotting with anti-P-Tyr and anti-topoI (human IgG1/IgG2; Topogen). Immunoprecipitation with human IgG as a control.
resulted in no detectable topoI in the precipitates (data not shown). B. Nuclear lysates from MCF-7/neo and MCF-7/c-Abl(K-R) cells were incubated at increasing concentrations with supercoiled (SC) DNA. No lysate was added to the control (last) lane. The DNA was analyzed by agarose gel electrophoresis (upper panel). The intensities of the SC bands were quantitated by image analysis and compared to that obtained for the no lysate (-) control (lower panel). MCF-7/neo (u); MCF-7/c-Abl(K-R) (◊). C. Lysates from wild-type c-Abl+/+ and c-Abl−/− cells were subjected to immunoprecipitation with anti-topoI. The immunoprecipitates were analyzed by immunoblotting with anti-P-Tyr and anti-topoI (left panel). Tyrosine phosphorylation of topoI was quantitated by densitometric scanning. The results are expressed as the mean±SD of four independent experiments (right panel). D. Nuclear lysates from c-Abl+/+ and c-Abl−/− cells were incubated at increasing concentrations with SC DNA. No lysate (-) was added to the control (last) lane. The DNA was analyzed by agarose gel electrophoresis (upper panel). The intensities of the SC DNA bands were quantitated by image analysis and compared to that obtained for the control (lower panel). The results are expressed as the mean±SD obtained for 4 separate experiments performed with the same lysate preparations. c-Abl+/+ (u); c-Abl−/− (◊).

**Fig. 5.** c-Abl phosphorylates topoI on Y268 in vitro. A. GST-topoI was incubated with purified c-Abl and [γ−32P]ATP for 30 min at 30°C. The reaction products were analyzed by SDS-PAGE and coomassie staining. A tryptic in gel digest of topoI was
fractionated by HPLC and $^{32}$p positive fractions were analyzed by MALDI-TOF-MS. M/Z 1762.33 represent phosphorylated y268 B. The $^{32}$p positive fraction was also analyzed by Edman sequencing and the eluted buffer from the sequencing were further analyzed for $^{32}$p activity.

**Fig. 6.** c-Abl-dependent phosphorylation and activation of topoI in the genotoxic stress response. c-Abl$^{+/+}$ and c-Abl$^{-/-}$ cells were treated with 5 Gy IR and harvested at 45 min. A. Lysates from control and irradiated cells were subjected to immunoprecipitation with anti-topoI. The immunoprecipitates were analyzed by immunoblotting with anti-P-Tyr and anti-topoI. B. Nuclear lysates from control and irradiated cells were analyzed in DNA relaxation assays. C. Image analysis of the gel shown in B. Control c-Abl$^{+/+}$ (□) and c-Abl$^{-/-}$ (△) cells. Irradiated c-Abl$^{+/+}$ (●) and c-Abl$^{-/-}$ (▲) cells.

**Fig. 7.** c-Abl phosphorylates topoI on Y268 in vitro and in cells. A. GST-topoI or the GST-topoI(Y268F) mutant was incubated with purified c-Abl and [$\gamma$-$^{32}$P]ATP for 30 min at 30°C. The reaction products were analyzed by SDS-PAGE and autoradiography. B. Purified GST-topoI and GST-topoI(Y268F) were incubated at increasing concentrations with SC DNA. The SC and R forms of DNA were analyzed by agarose gel electrophoresis (upper panel). Image analysis of the gel is shown for GST-topoI (□) and GST-topoI(Y268F) (△) (lower panel). C. GST-topoI(Y268F) was incubated in the absence and presence of purified c-Abl and ATP. The GST-
topoI(Y268F) proteins were purified and incubated at increasing concentrations with SC DNA. The DNA was analyzed by agarose gel electrophoresis (upper panel). The SC DNA was quantitated by image analysis for GST-topoI(Y268F) incubated without (△) and with (▲) c-Abl (lower panel).

**Fig. 8.** c-Abl−/−, as compared to c-Abl+/+, cells are less sensitive to CPT-induced PLDB and apoptosis. A. c-Abl+/+ (m) and c-Abl−/− (o) cells were labeled with [3H]thymidine, treated with the indicated concentrations of CPT for 30 min and then assayed for PLDB. The results are expressed as the percentage of PLDB (mean ± SE) compared to that of control cells as determined from three separate experiments each performed in duplicate. B. c-Abl+/+, c-Abl−/− and c-Abl+ cells were labeled with [3H]10 µM CPT for 1 h and then assayed for DNA single-strand breaks (SSB) by alkaline elution. The results are expressed as SSB frequency in rad equivalents (73). C. c-Abl+/+, c-Abl−/− and c-Abl+ cells were incubated with 10 µM CPT for 30 min and harvested at 24 h. Control and treated cells were analyzed for sub-G1 DNA by flow cytometry. Sub-G1 DNA content: control c-Abl+/+, 1.7%; CPT-treated c-Abl+/+, 25.3%; control c-Abl−/−, 0.1%; and CPT-treated c-Abl−/−, 0.5%. Control c-Abl+, 0.3% and CPT-treated c-Abl+, 25.0%.
**Fig. 1**

A. 

```
| Lysate | anti-c-Abl | IgG |
|--------|------------|-----|
| kDa 175 |            |     |
| kDa 83  |            |     |
```

IB: anti-Topol

B. 

```
| Lysate | anti-Topol | IgG |
|--------|------------|-----|
| kDa 175 |            |     |
| kDa 83  |            |     |
```

IB: anti-c-Abl

C. 

```
| Lysate | GST | GST-Grb-2-SH3 | GST-c-Abl-SH3 |
|--------|-----|---------------|---------------|
|        |     |               |               |
```

IB: anti-Topol

D. 

```
| GST-Topol |
|-----------|
| 211-765  |
| 1-250    |
| 1-765    |
```

IB: anti-c-Abl

E. 

```
| c-Abl | GST+c-Abl | GST-topol+c-Abl |
|-------|-----------|-----------------|
| kDa 175 |         |                  |
| kDa 83  |         |                  |
```

IB: anti-c-Abl
Fig. 2B

ERINKLESNLN

Calculated [M+H]+ = 1287.46
Calculated [M+2H]2+ = 644.23
Experimental [M+2H]2+ = 644.17
C.

**IP: anti-Topol**

- c-Abl +/+ (175 kDa)
- c-Abl -/- (175 kDa)
- 83 kDa

**IB: anti-P-Tyr**

- 175 kDa (Topol)
- 83 kDa (Topol)

**IB: anti-Topol**

**Topol (pY) % Control**

- c-Abl +/+ (150%)
- c-Abl -/- (50%)

---

Fig. 4C
Fig. 4D

D.

![Image of gel electrophoresis and graph showing the effect of nuclear protein on supercoiled DNA].

- c-Abl<sup>+/+</sup>
- c-Abl<sup>−/−</sup>
- Lysate
- R
- SC

Graph shows the percentage of supercoiled DNA (% Supercoiled DNA) against nuclear protein (μg) with data points at 3, 6, 9, 12, and 15 μg.
Fig. 6

A. IP: anti-Topol

IB: anti-P-Tyr

IB: anti-Topol

B. Control IR

c-Abl+/+ c-Abl−/− c-Abl+/+ c-Abl−/−

(−) Lysate

C. % Supercoiled DNA

% Supercoiled DNA

0 20 40 60 80 100

Nuclear Protein (μg)

0 4 8 12 16 20 24
Fig. 7BC

B.

Topol

Topol (Y268F)

R

SC

C.

Topol (Y268F)

Topol (Y268F) + c-Abl

R

SC

% Supercoiled DNA

0 10 20 30

Topo I (ng)

% Supercoiled DNA

5 10 15 20 25

Topo I (ng)
Phosphorylation of DNA topoisomerase I by the c-Abl tyrosine kinase confers camptothecin sensitivity
Donghui Yu, Ehsan Khan, Md Abdul Khaleque, James Lee, Gary Laco, Glend Kohlhagen, Surender Kharbanda, Yung-Chi ChengHENG, Yves Pommier and Ajit Bharti

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