Modulation of Human DNA Topoisomerase IIα Function by Interaction with 14-3-3ε

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Human DNA topoisomerase IIα (topo II), a ubiquitous nuclear enzyme, is essential for normal and neoplastic cellular proliferation and survival. Several common anticancer drugs exert their cytotoxic effects through interaction with topo II. In experimental systems, altered topo II expression has been associated with the appearance of drug resistance. This mechanism, however, does not adequately account for clinical cases of resistance to topo II-directed drugs. Modulation by protein-protein interactions represents one mechanism of topo II regulation that has not been extensively defined. Our laboratory has identified 14-3-3ε as a topo II-interacting protein. In this study, glutathione S-transferase co-precipitation, affinity column chromatography, and immunoprecipitations confirm the authenticity of these interactions. Three assays evaluate the impact of 14-3-3ε on distinct topo II functional properties. Using both a modified alkaline comet assay and a DNA cleavage assay, we demonstrate that 14-3-3ε negatively affects the ability of the chemotherapeutic, etoposide, to trap topo II in cleavable complexes with DNA, thereby preventing DNA strand breaks. By electrophoretic mobility shift assay, this appears to be due to reduced DNA binding activity. The association of topo II with 14-3-3 proteins does not extend to all 14-3-3 isoforms. No protein interaction or disruption of topo II function was observed with 14-3-3ε.

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Experimental Procedures

Mammalian Cell Culture—HeLa human cervical carcinoma cells, HL-60 human promyelocytic leukemia cells, and CCRF/CEM human acute lymphoblastic leukemia cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), and HL-60 and CCRF/CEM cells were maintained as described in Ref. 2000 in RPMI 1640 medium (Life Technologies, Inc.). Both media were supplemented with 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

DNA Expression Library Screening for TIFs—To identify unknown protein interactive partners of topo II, approximately 1 × 10⁸ plaques of a HeLa cDNA library in αgt11 (CLONTECH, Palo Alto, CA) were plated at the density of 2000 in RPMI 1640 medium (Life Technologies, Inc.). Induction was accomplished by overlaying plates with 132-ml nutrient-cellulose filters soaked in 10 ml isopropylthio-β-galactoside. Filter lifts were subjected to a protein denaturing/renaturing protocol (37), blocked and probed as described previously (9), but using 125I-human topo II (amino acids 857–1448) as the interaction probe. Expression and purification of this recombinant protein have been detailed elsewhere (9).

Filters were washed extensively prior to autoradiography. Putative positive clones were plated for two additional cycles of screening until plaque purification was achieved. Selected cDNA clones were obtained by polymerase chain reaction using αgt11 primers and the PCR products directly cloned in the T-tailed sequencing vector, pCRII (Invitrogen, Carlsbad, CA). One TIP clone was identical to the DNA sequence corresponding to amino acids 126–255 (terminus) of the epsilon form of human topo II protein (15). Full-length cDNA was obtained via polymerase chain reaction using a dilution of HeLa cDNA library as the template and was subcloned in frame with glutathione transferase (GST) in pGEX-2TK (Amersham Pharmacia Biotech) to enable prokaryotic expression and affinity purification of the resulting chimeric protein (GST14-3-3e).

GST Co-precipitation Assays—The co-precipitation method of Conklin et al. (38) was employed. Briefly, 10–200 μg of purified GST14-3-3e peptides or GST alone was incubated with 20 μg of topo II peptide (amino acids 857–1448) in PBS containing 0.5 mM Triton X-100 and 0.1% BSA for 16 h at 4 °C. In experiments where interactions of full-length proteins were investigated, CCRF/CEM nuclear extract was used as the topo II source. Portions of the nuclear extract were treated with calf intestinal alkaline phosphatase (Life Technologies, Inc.) (1 unit/μg of extract) prior to incubation with GST fusion proteins in a buffer containing 40 mM Hepes, 100 mM NaCl, 0.5 mM EDTA, 5 mM MgCl₂, and 0.05% Nonidet P-40. Complexes were subsequently precipitated by the addition of 10 μl of a 50% slurry of glutathione-Sepharose (Amersham Pharmacia Biotech) and incubated on ice. Beads were allowed to settle, washed three times in binding buffer, then heated to 95 °C in 2× Laemmli SDS sample buffer. Following SDS-PAGE and electrotransfer, blots were probed with a polyclonal anti-topo II antibody as a control. Following a 2-h incubation on ice, complexes were precipitated by a 30-min incubation with another 20 μl of Protein A/Protein G-agarose. Complexes were washed three times with 500 μl of RIPA, then reloaded by boiling for 5 min in 2× Laemmli SDS sample buffer, followed by immunoblotting for topo II.

For affinity chromatography, the large C-terminal fragment of topo II (amino acids 280–623 of XPR1/X3) was expressed as a polyhistidine fusion protein as described previously (9) and purified by nickel-agarose affinity chromatography. One aliquot of purified protein (250 μg) was phosphorylated with 100 units of recombinant, human casein kinase II (New England Biolabs, Beverly, MA) in 7 ml of 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.2 mM ATP for 30 min at 30 °C. Control topo II protein was incubated under similar conditions but without the addition of kinase. Separate 400-μl bed volumes of fresh nickel-agarose (Promobeads, InviTrogen, Carlsbad, CA) were loaded into individual minicolumns and bound with 250 μg of BSA, control topo II protein, or casein kinase II-phosphorylated topo II protein. Columns were then washed with 10 bed volumes of 20 mM sodium phosphate, pH 7.8, 500 mM NaCl. A RIPA extract of CCRF/CEM cells (5 μl total protein) was then applied to the column, eluted 1.7 in PBS, and reapplied to the column three more times. The columns were washed extensively with 40 bed volumes of PBS, and bound proteins were eluted with multiple 500-μl aliquots of 0.5% SDS in 12.5 mM Tris-HCl, pH 6.8. Aliquots of each fraction were supplemented with 2-mercaptoethanol, boiled, and resolved on a 12% SDS-PAGE prior to immunoblotting with a broad spectrum 14-3-3 antiserum (K-19).

Alkaline Naked Comet Assay—A modified alkaline comet assay using isolated HL-60 nuclei was performed to assess the effect of 14-3-3 on topoisomerase II-mediated DNA damage (39). HL-60 nuclei were prepared from logaritomically growing cultures by standard techniques and incubated in PBS in the presence or absence of 30 μM etoposide and GST, GST14-3-3e, or GST14-3-3b protein (0.16–20 μg/ml). Nuclei were incubated on ice for 90 min and then subjected to a standard electrophoretic comet protocol (40). After staining with ethidium bromide (20 μg/ml), nuclei were quantified by fluorescence microscopy for total nuclear and tail length.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotides corresponding to residues 87–126 of pBR322, a strong topo II binding site (41), were purified and end-labeled with [³²P]dCTP. Protein extracts (500 mM NaCl extraction) were prepared from logaritomically growing HeLa cells as described previously (16) and using Complete-protease inhibitor (Roche Molecular Biochemicals). Incubations of the binding site with nuclear protein extract were carried out on ice in a 25-μl reaction volume containing 50 mM KCl, 20 mM Tris (pH 7.6), 2 mM MgCl₂, 1 mM EDTA, 10% glycerol, 2.5 μg of BSA, and 0.1 μg of poly(dI-dC)/d(dI-dC) (Amersham Pharmacia Biotech). Free and bound oligonucleotide were separated by electrophoresis through a 4% nondenaturing polyacrylamide gel in 0.25× Tris borate-EDTA buffer. For competition EMSA, molar excesses of unlabelled competitor oligonucleotide were premixed with the [³²P]-labeled binding site oligonucleotide before the addition of reaction buffer and nuclear protein extract. For supershift EMSA, purified topo II protein was added 30 min after the initiation of the binding reaction. The mixture was incubated on ice for an additional 30 min prior to electrophoresis.

DNA Cleavage Assay—DNA cleavage assays were carried out essentially as described in Ref. 42. Typically, reactions contained 300 ng of pBR322 and were initiated by the addition of 6 units of purified human topo II (Topogen, Columbus, OH) and incubated at 37 °C for 8 min. Reactions were terminated by the addition of SDS (0.5% final concentration) and incubated with protease K (200 μg/ml) at 56 °C for 1 h. Reaction components were resolved at 20 V for 16 h on a 1.3% agarose gel containing 0.7 μg/ml ethidium bromide to allow the relaxed closed-circular plasmid to run with greater mobility than supercoiled DNA so as to distinguish it from open, nicked-circular DNA (42).

Results

14-3-3e is a Topo II-interacting Protein—To clone and identify TIFs, a αgt11-HeLa cell cDNA library was screened with radiiodinated topo IIα-(857–1448). Four open reading frames were identified that survived three rounds of screening and plaque purification. Three clones corresponded to amino acids 280–623 of XPR1/X3, a membrane-bound receptor for xenotropic and polytropic murine leukemia viruses (43, 44), and are not considered further here. The fourth open reading frame clone was 100% identical to the DNA sequence encoding amino acids 126–255 of the epsilon isofrom of human 14-3-3 protein. 14-3-3e Interacts with Topo II in Solution—To verify the authenticity of the 14-3-3e/topo II interaction, expression vectors encoding GST in frame with the N terminus of 14-3-3e (or fragment thereof) were generated and tested for the ability to co-purify with topo II from extracts (Fig. 1). Both the full-length GST14-3-3e and a C-terminal fragment (amino acids 126–255, interacted with topo II-(857–1448), while GST alone did not precipitate any of the recombinant topo II peptide (Fig. 1A). Conversely, the topo II-(857–1448) fragment, containing a polyhistidine tag, was immobilized on a nickel-chelating column and was tested for the ability to adsorb endogenous 14-3-3 proteins using CCRF/CEM RIPA extract as the source. When
compared with a BSA control column, the topo II column selectively co-purified a protein of 30–32 kDa that reacted with a broad spectrum antiserum to all isoforms (Fig. 1B). Phosphorylation of the top II (857–1448) fragment with casein kinase II prior to immobilization on the nickel-chelating column greatly enhanced the co-purification of the 14-3-3-immunoreactive 30–32-kDa protein (Fig. 1B), relative to either BSA or unphosphorylated top II (857–1448).

As the above experiments were carried out using a recombinant top II peptide, we examined whether GST14-3-3ε was able to interact with full-length 170-kDa top II from nuclear protein extracts. Using nuclear protein extracts from CCRF/CEM cells as a source of 170-kDa top II, we determined that GST14-3-3ε, but not GST, could interact with full-length topo II (Fig. 1C). This association of topo II with 14-3-3 proteins does not extend to all isoforms, as no interaction was observed with a GST14-3-3ζ fusion protein even though comparable amounts of protein were used (Fig. 1C). Using an alkaline phosphatase treatment previously demonstrated to remove more than 80% of the phospholinkages on topo II (45), we demonstrated that, while the GST14-3-3ε fusion protein preferentially interacts with a phosphorylated form of topo II (Fig. 1C), dephosphoryl-
Functional Interaction of DNA Topo IIα and 14-3-3ε

Fig. 2. GST14-3-3ε protects HL-60 nuclei from etoposide-mediated DNA strand breaks. Panel A, isolated HL-60 nuclei were incubated in the absence (hatched bars) or presence (open bars) of 30 μM etoposide and GST, GST14-3-3ε, or GST14-3-3ε protein (10 μg/ml) embedded in agarose, and electrophoresed. After staining with ethidium bromide (20 μg/ml), nuclei were quantified by fluorescence microscopy for comet (nuclear plus tail) length. Comet lengths are expressed relative to untreated HL-60 controls. The means (± S.D.) of 30 comets are shown for each data point. The relative length of comets in the presence of 10 μg/ml GST14-3-3ε and 30 μM etoposide differed significantly from other etoposide-exposed nuclei (*, p < 0.005 one-way analysis of variance with a post hoc test) but did not differ significantly from untreated controls. Panel B, relative comet length of untreated (closed symbols, hatched region) or etoposide-treated (30 μM) (open symbols) HL-60 nuclei in the presence of GST (10 μg/ml) or increasing concentrations of GST14-3-3ε (0.16, 0.8, 4.0, and 20.0 μg/ml). The means of 30 comets are shown for each data point. Nuclear lengths differing significantly from untreated controls are indicated (*, p < 0.005).

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atation abrogates the interaction.

Finally, we investigated the ability of 14-3-3 antisera to co-immunoprecipitate 170-kDa topo II from CCRF-CEM cell extracts, using standard methods but with the addition of high stringency washing conditions with RIPA (instead of the low salt, detergent-lacking washes used commonly) (Fig. 1D). Using antisera recognizing either all 14-3-3 isoforms or only the ε isoform, a topo II immunoreactive band in immunoprecipitates migrating at 170 kDa was observed when using 500 μg of cell extract. The authenticity of this interaction is emphasized by the fact that this experiment was performed without overexpression of either protein, but rather using the endogenous levels of each protein normally present in CCRF-CEM cells. Despite the fact that 14-3-3 is capable of binding several other proteins in mammalian cells, the interaction with topo II was of significant abundance to be detected in this assay.

14-3-3ε Reduces Etoposide-mediated DNA Damage—To investigate the effect of 14-3-3ε on topo II in the intact nucleus, we modified the alkaline comet assay using purified HL-60 nuclei (39). Co-incubation of nuclei with GST14-3-3ε significantly protected the nuclei from etoposide-mediated DNA damage (Fig. 2A), while addition of GST or GST14-3-3ε provided no protection from DNA strand breakage. In the absence of etoposide-induced DNA damage, co-incubation of HL-60 nuclei with GST, GST14-3-3ε, or GST14-3-3ε had no effect on observed nuclear length (Fig. 2A). The protection from etoposide-mediated DNA damage by GST14-3-3ε was dose-dependent with significant protection from DNA strand breaks in the presence of 0.8 μg/ml protein (Fig. 2B).

14-3-3ε Abrogates Topo II DNA Binding Activity—To evaluate the effect of 14-3-3ε on topo II DNA binding activity, EMSAs were carried out using a radiolabeled double-stranded oligonucleotide containing a strong topo II binding site. Competition EMSA, using increasing molar excesses of unlabeled oligonucleotide representing the topo II binding site and an oligonucleotide of unrelated sequence, demonstrated that the predominant observed DNA-protein interaction is sequence-specific (Fig. 3A). Addition of an anti-topo IIα antisera produced a supershifted complex, indicating the involvement of topo II in the protein-DNA complex (Fig. 3B). Incubation of the nuclear extract with a nonspecific antiserum produced neither a supershifted complex nor a reduction in the intensity of the specific DNA-protein complex (Fig. 3B). GST14-3-3ε was added to the binding reaction to evaluate its effect on topo II DNA binding activity. Addition of increasing amounts of GST14-3-3ε abrogated topo II DNA binding activity (Fig. 3C). This effect was specific for 14-3-3ε, as the addition of increasing amounts of GST alone or GST14-3-3ε had no effect on the topo II-DNA interaction (Fig. 3C).

14-3-3ε Decreases Etoposide-stabilized Cleavable Complex
Formation—To examine whether 14-3-3e would alter the ability of etoposide to trap topo II in cleavable complexes, a plasmid DNA cleavage assay was performed (Fig. 4). This assay, designed to evaluate the catalytic activity of topo II, demonstrates that 14-3-3e negatively affects the ability of etoposide to stabilize topo II in covalent complexes with DNA, as demonstrated by the reduction in intensity of the linear DNA band (Fig. 4). The inhibitory effect observed was specific for GST14-3-3e, as neither GST alone nor GST14-3-3σ had an effect on the cleavage-religation equilibrium.

**DISCUSSION**

Through a HeLa cDNA expression library screen, we identified 14-3-3e as a topo II-interacting protein. We have verified the authenticity of this interaction by demonstrating that (a) recombinant GST14-3-3e is able to interact in solution with either a recombinant topo II peptide (857–1448) or full-length topo II in nuclear protein extracts, (b) recombinant topo II-(857–1448) immobilized on a column interacts with 14-3-3 proteins in a whole cell extract, and (c) two antisera recognizing 14-3-3 proteins are able to precipitate full length topo II from cellular extract without the need for overexpression of either protein. Consistent with reports of the preferential binding of 14-3-3 to phosphorylated proteins, the interaction between 14-3-3e and topo II is significantly enhanced by prior phosphorylation of a recombinant topo II fragment and attenuated by phosphatase pretreatment of the nuclear protein extract.

It is well established that topo II, both in vitro and in vivo, is associated with and phosphorylated by casein kinase II (46–48), although the precise residues phosphorylated in vivo remain to be defined. In addition to phosphorylating topo II,
that the limited distribution of 14-3-3 e
Two consensus sequences for 14-3-3 binding have been identified
lute requirement (51), the interaction of 14-3-3 with its targets is
or not cell cycle variations in the level of site-specific phospho-
plicated by the use of topo II from different species and whether
consistent finding (50). The interpretation of these studies is com-
regulate the activity of this enzyme, although this remains a
While the amino acid sequences of the seven identified 14-3-3
exclude specific isoforms as candidate interacting proteins.
with multiple 14-3-3 isoforms (20, 22, 56), other 14-3-3 protein
isoforms throughout evolution suggests that they may have
evolved distinct, non-redundant functions. Although some pro-
even though not all reports were experimentally
whether topo II interacts with other 14-3-3 isoforms.
Although phosphorylation of its protein partner is not an abso-
lute requirement (51), the interaction of 14-3-3 with its targets is
most often mediated by the recognition of a phosphoserine motif.
Two consensus sequences for 14-3-3 binding have been identified
(52, 53). Examination of the topo II sequence reveals a single
potential 14-3-3 binding site (1028RGYDSDP)888 that also contains
within it a cas kinase II consensus phosphorylation site (54).
Whether this site is phosphorylated by cas kinase II or contrib-
utes to the interaction with 14-3-3 e is under investigation.

To establish the functional consequences of these interac-
tions, we have used three independent assays to evaluate the
impact of 14-3-3 e on distinct topo II activities. Using both a
modified alkaline comet assay with isolated HL-60 nuclei and a
plasmid DNA cleavage assay, we demonstrated that 14-3-3 e negatively affects the ability of etoposide to trap topo II in
cleavable complexes, thereby preventing the induction of DNA
strand breaks. By EMSA, it is demonstrated that this appears
to be due to reduced DNA binding activity. Whether 14-3-3 e
induces a change in the preferred DNA binding sequence for
topo II remains to be tested. Taken together, these findings
suggest that the effect of 14-3-3 e on topo II activity be evalu-
at in intact cells, since the consequence of this interaction is
reminiscent of topo II inhibition as a result of physical associ-
ation with the tumor suppressor gene product, Rb (10).

Although an extensive range of studies investigating 14-3-3 proteins have been published, few papers characterize more than one isoform, while even fewer reports experimentally exclude specific isoforms as candidate interacting proteins. While the amino acid sequences of the seven identified 14-3-3 isoforms are highly conserved (55), the retention of multiple isoforms throughout evolution suggests that they may have evolved distinct, non-redundant functions. Although some proteins, such as Raf, may be promiscuous in their association with multiple 14-3-3 isoforms (20, 22, 56), other 14-3-3 protein partners demonstrate a degree of selectivity (31, 57). This report demonstrates that 14-3-3 e, but not 14-3-3 s, interacts with topo II, thereby modulating several in vitro measures of topo II function. 14-3-3 e is an atypical isoform, being more closely related to yeast and plant 14-3-3 proteins than to other mammalian isoforms (55, 58). This early divergence may have given rise to distinct roles for 14-3-3 e. It should be considered that the limited distribution of 14-3-3 s to cells of epithelial
origin, in contrast with the ubiquitous distribution of 14-3-3 e, may also play a role in influencing their functions (19, 59).

Although it is clear that topo II interacts with 14-3-3 e (and 14-3-3 s,2) but not with 14-3-3 s, it is not currently known whether topo II interacts with other 14-3-3 isoforms.

There are several hypotheses as to the mechanism of 14-3-
3 e-mediated disruption of topo II function. First, dimerization is crucial for topo II DNA-binding and function. Recently, two sequences in human topo II (spanning amino acids 1053–1069
and 1124–1143) were identified to be crucial for this dimeriza-
tion, with disruption of either sequence leading to a loss of capacity to dimerize (60). It is of note that the candidate 14-3-3 e binding site falls between these two sequences. Second, numerous 14-3-3 isoforms, including 14-3-3 s, have been recently described as mediating subcellular localization of their protein partners, including protein kinase A (36), and, in response to DNA damage, the cell cycle regulatory protein cdc25, thereby inducing cell cycle arrest (32–35). Hypothetically, the interaction between 14-3-3 e and topo II could modulate the subcellular
localization of topo II, either during phases of the cell cycle, or in response to DNA damage thereby modulating cellular sensitivity to topo II poisons. Whether the binding of 14-3-3 e disrupts topo II homodimerization, modulates topo II localization, or functions through a mechanism yet to be considered remains the focus of our ongoing investigation.

In summary, we have identified 14-3-3 e as a topo II-inter-
acting protein. Our finding that this interaction with topo II does not extend to all 14-3-3 isoforms, as demonstrated by the lack of interaction with 14-3-3 s, is one of a small, but growing, number of reports that hint at functional specificity within this well conserved protein family. This observation suggests that evolutionary retention of multiple isoforms may be attributable to hereto unappreciated distinct and non-redundant functions for individual isoforms or subsets thereof. The demonstration that 14-3-3 e disrupts topo II/DNA interaction and reduces eto-
poside-mediated cleavable complex formation and DNA strand
breaks has significant implications for the cellular sensitivity to topo II-directed chemotherapeutics.

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