Serine Phosphorylation-Dependent Coregulation of Topoisomerase I by the p14ARF Tumor Suppressor†

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ABSTRACT: p14ARF (ARF) and topoisomerase I play central roles in cancer and have recently been shown to interact. The interaction activates topoisomerase I, an important target for camptothecin-like chemotherapeutic drugs, but the regulation of the interaction is poorly understood. We have used the H358 and H23 lung cancer cell lines and purified recombinant human topoisomerase I to demonstrate that the ARF/topoisomerase I interaction is regulated by topoisomerase I serine phosphorylation, a modification that regulates topoisomerase I activity. Both cell lines express wild-type ARF and topoisomerase I proteins at equivalent levels, but H23 topoisomerase I, unlike that of H358 cells, is largely devoid of serine phosphorylation, has low activity, and complexes poorly with ARF. The ability of H23 topoisomerase I to complex with ARF can be restored by treatment with the serine kinase, casein kinase II. Consistent with these observations, we show that the response of H23 cells to camptothecin treatment is unaffected by changes in intracellular levels of ARF. However, in H358 and PC-3 cells, which express a serine phosphorylated topoisomerase I that complexes with ARF, ectopic overexpression of ARF causes sensitization to camptothecin, and siRNA-mediated down-regulation of endogenous ARF causes desensitization to camptothecin. These biological responses correlate with increased and decreased levels, respectively, of ARF/topoisomerase I complex and DNA-bound topoisomerase I. Thus, ARF is a serine phosphorylation-dependent coregulator of topoisomerase I in vivo, and it regulates cellular sensitivity to camptothecin by interacting with topoisomerase I. Certain cancer associated defects affecting ARF/topoisomerase I complex formation could contribute to cellular resistance to camptothecin.

The p14ARF tumor suppressor (hereafter referred to as ARF[1] for alternate reading frame) initially attracted attention as one of the most frequently altered genes in human cancer (*1*) and a key positive regulator of the p53 tumor suppressor pathway. The induction of this pathway in response to oncogenic hyperproliferative signals has been shown to provide a critical barrier to the outgrowth of cells with activated oncogenes (*2*−*4*).

In addition to its role in the p53 pathway, ARF now appears to play additional roles in cellular regulation, possibly involving nucleolar activity (*5*−*7*). ARF has recently been shown to interact with the nucleolar enzyme topoisomerase I (hereafter referred to as topo I) (*8*, *9*), an important target for cancer chemotherapeutics located in the internucleosomal regions of ribosomal RNA chromatin (*10*). The interaction of purified ARF and topo I proteins requires the C-terminal domain of ARF (*8*), a region that is not required for ARF's p53-dependent activity, and results in stimulation of topo I activity (*9*). Because both ARF and topo I proteins play key roles in cancer, their interaction is of considerable interest. However, the regulation of complex formation between the ARF and topo I is poorly understood and the role of the complex in the cellular response to topo I targeted drugs has not been investigated.

Topo I is an essential cellular enzyme that catalyzes the stepwise change in the linking number of supercoiled DNA needed to relieve torsional stress on DNA during replication and transcription (*11*, *12*). The enzymatic reaction involves the introduction of a transient single strand break in the DNA double helix, via an intermediate covalent complex between the enzyme and DNA ("cleavable complex"). This allows passage of the other strand, followed by resealing of the break and release of the enzyme. The plant alkaloid camptothecin stabilizes the cleavable complex between topo I and DNA, thereby preventing the religation of the single strand break (*11*). In cells treated with camptothecin, the nick becomes a lethal double strand break upon passage of the replication fork (*13*). This mechanism accounts for the therapeutic effects of camptothecin-related chemotherapeutic drugs, including ironotecan (CPT-11, Camptosar) and topotecan (Hycamtin), which have proven to be highly effective for

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[1] Abbreviations: ARF, alternate reading frame; topo I, topoisomerase I; siRNA, small inhibitor RNA; MTS, 3-(4,5′-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt; AP, alkaline phosphatase; CKII, casein kinase II; RIPA, reticulocyte immunoprecipitation assay.

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the treatment of a variety of cancers (*14*). The clinical efficacy of these agents is limited by the acquisition of therapy resistance by the tumor, however, a process that may involve a variety of mechanisms (*15*, *16*), including down-regulation of topo I activity (*17*). A better understanding of the factors that regulate the formation of the ARF/topo I complex could suggest strategies to improve cellular responses to camptothecin-related drugs.

In this study, we have used lung cancer cell lines with differing abilities to form ARF/topo I complexes, as well as purified human topoisomerase I, to demonstrate that the interaction of these two proteins requires topo I serine phosphorylation, a positive regulator of topo I activity. We provide evidence that ARF regulates cellular sensitivity to camptothecin by interacting with topo I and promoting its association with DNA. The results have implications for an expanded role of ARF in cellular regulation and in the cellular response to topo I-targeted therapies and suggest that certain cancer-associated defects in topo I that impair its ability to interact with ARF could contribute to therapy resistance.

EXPERIMENTAL PROCEDURES

Cell Lines. H358 and H23 non-small-cell lung cancer cells and PC-3 prostate cancer cells were obtained from American Type Culture Collection (Rockville, MD). All cell lines express endogenous ARF (*6*) but have abnormalities in p53 function (*6*, *18*). Cell lines were maintained as described previously (*19*).

Vectors. The Adp14 vector encoding full-length ARF, the Ad1β vector encoding the 64-amino acid residue N-terminal domain of ARF (ARF N-term), and vector treatment conditions have been described (*6*, *19*). Equal titers of Adp14 and Ad1β were confirmed by RT-PCR to produce equivalent levels of ARF and ARF N-term message. The AdLuc control vector encoding firefly luciferase was provided by Introgen Therapeutics, Inc. The Adp53 vector was purchased from Vector BioLabs, Philadelphia, PA. An siRNA expression plasmid specific for the exon 2-encoded region of ARF (pKD-Ink4a-v2) and a negative control siRNA expression plasmid (pKD-NegCon-v1) were purchased from Upstate (Lake Placid, NY) and transfected into cells using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. An siRNA to the exon 1β region of ARF (sense sequence, 5′-GGGUUUUCGUGGUUCACAUtt-3′; antisense sequence, 5′-AUGUGAACCACGAAAAC-CCtc-3′) was purchased from Ambion, Inc. (Austin, TX).

Cell Viability Assays. Cells at about 60% confluency in 12-well or 24-well plates in Dulbecco's modified Eagles medium (DMEM) plus 2% fetal bovine serum were treated for 4 h with replication-defective adenoviral vectors at a multiplicity of infection of 20 or with 2 μg siRNA expression plasmid using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following vector treatments, cells were replated at 2000 cells per well in 96-well plates in complete growth medium and allowed to attach overnight. The next day (24-h after vector treatment), triplicate wells of cells were treated with increasing doses of camptothecin (Sigma, St Louis, MO) for 24 h, and viability was scored 5 days after vector treatment by the MTS [3-(4,5′-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (Promega Corp., Madison, WI)] bioconversion assay as previously described (*6*). Under these conditions, control, untreated cells remained in exponential growth.

Pull-Down Assays. Thioredoxin fusion proteins for full length human fibroblast ARF and the ARF N-term domain encoded by ARF's first exon were constructed using the pET32a vector, expressed in IPTG-induced *Escherichia coli* BL21(DE3) (Novagen), and purified according to the manufacturer's protocol (Novagen, Madison, WI). Fusion proteins were immobilized on nickel−NTA−agarose (Qiagen, Valencia, CA) and incubated with cell lysates (precleared of nonspecific binding proteins by incubation with nickel−NTA−agarose) prepared in RIPA (reticulocyte immunoprecipitation assay) buffer [10 mM sodium phosphate (pH 7), 0.15 M NaCl, 0.1% SDS, 1% NP40, 1% sodium deoxycholate, 1 mM phenymethylsulfonyl fluoride (PMSF) and complete protease inhibitors (Roche, Nutley, NJ)]. This treatment results in complete lysis of cells by disrupting cellular and nuclear membranes. The bound fraction was eluted with 50 mM EDTA and analyzed by SDS−PAGE/Western blot (immunoblot), as described previously (*6*). Protein concentrations in lysates were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions.

Sequence Analysis. PCR products of H23 cell cDNA, representing 500−600-base pair overlapping portions of H23 cell topo I, were subcloned into the plasmid vector pCR2.1-TOPO using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced. A 539-base pair PCR product encompassing the entire ARF coding region of H23 or H358 cDNA was directly sequenced. Sequences were carried out by BatJ, Inc. (San Diego, CA).

Co-Immunoprecipitation (IP)/Western Blot (Immunoblot). DNAse I-solubilized nuclear extracts [10 mM Hepes (pH 7.5), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM PMSF, protease inhibitor pellet (Roche, Nutley, NJ)], which contained the bulk of cellular topo I, were prepared according to ref *20* and subjected to immunoprecipitation, following our previously described procedure (*21*). We found that high-salt-extracted nuclei (using 0.4 M NaCl buffer described in ref *22*; see topo I assays, below) and DNAse I-solubilized nuclei were similar with respect to topo I and ARF recovery; however, DNAse I solubilization avoided high salt concentrations that might disrupt complexes. Where bound and unbound fractions were to be compared, the extracts were subjected to two successive immunoprecipitations, to completely deplete extracts of immunoreactive material. The pooled immunoreactive ("bound") material from the first and second treatments was subjected to SDS−PAGE/Western blot (immunoblot) analysis. The unbound material was concentrated by precipitation with 5 volumes of acetone, prior to resuspension in sample buffer for SDS−PAGE/Western analysis. Co-immunoprecipitations with purified baculovirus-expressed human topoisomerase I (TopoGEN, Port Orange, FL) and purified ARF fusion protein were carried out using 10 pmol (1 μg) of topoisomerase I and 10 pmol (0.14 μg) of ARF fusion protein. Antibodies were goat polyclonal anti-topo I (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-full length ARF (Zymed Laboratories, Inc., South San Francisco, CA), mouse mono-

clonal anti-phosphoserine (Sigma, St. Louis, MO), and mouse monoclonal anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). All primary antibodies were used at 1:100 for Western blots. Secondary antibodies for Western immunoblots were goat anti-rabbit, goat anti-mouse, and donkey anti-goat (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA) and were used at 1:1000. Protein concentrations in lysates were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions.

Topo I Assays. High-salt nuclear extracts (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10% glycerol, and complete protease inhibitors) were prepared as described (*22*) and assayed using the Topo I Assay Kit (TopoGEN, Port Orange, FL), according to the manufacturer's instructions. Briefly, 0.125 μg of supercoiled plasmid DNA was incubated with 0−1.3 μg of nuclear extract for 30 min at 37 °C. Assays were also carried out with purified human topo I expressed in a baculovirus/insect cell system (TopoGEN), using 0.5 units of topo I per reaction. The TopoGEN enzyme was supplied at a concentration of 10 units per μL, with 1 unit being defined as the amount needed to convert 0.1−0.5 μg of supercoil DNA to its relaxed form in 30 min at 37 °C. The reaction was stopped by adding stop loading dye supplied in the kit and electrophoresed on a 1% agarose/TAE (10 mM Tris-acetate/1 mM EDTA) gel, and the reaction products were visualized by ethidium bromide staining (0.5 μg/mL). Supercoil band intensities in topo I-treated samples were analyzed digitally using a Kodak digital camera and analysis software and expressed as a percentage of the supercoil band intensity in the control, untreated sample. Some assays were carried out in the presence of ARF or ARF1β fusion proteins (3, 9, 27 ng).

Alkaline Phosphatase and Casein Kinase II Treatments. Dephosphorylation of high-salt nuclear extracts (prepared as for topo I assays and as described in ref *22*) or purified baculovirus-expressed human topoisomerase I (TopoGEN, Port Orange, FL) was carried out using alkaline phosphatase (Sigma, St. Louis, MO) as described (*23*). For co-immunoprecipitations, where high salt needed to be avoided, dephosphorylations were carried out with DNAse I-solubilized nuclear extracts [as described in ref *20*; see Co-Immunoprecipitation/Western Blot (Immunoblot) section]. Serine phosphorylation of dephosphorylated nuclear extracts or purified topo I was carried out with casein kinase II (Promega, Madison, WI), following the manufacturer's protocol.

Quantitation of Topo I/DNA Complexes by Co-precipitation. Adenoviral vector-treated H358 cells (2×10^5) were treated 24 h later with 1.2 μCi [^{3}H]thymidine (NEN Lifescience Products, MA) overnight, followed by a 1-h incubation in nonradioactive medium and a 25-min incubation in 0.08 μM camptothecin to cross-link topo I to DNA. Cells were washed in cold PBS, and cross-linked topo I/DNA complexes were recovered using the K^+-SDS method (*24*), resuspended in water, and subjected to liquid scintillation counting.

Quantitation of Topo I/DNA Complexes by Immunodepletion. Adp14-treated H358 cells (2×10^6) were treated 48 h later with 0.08 μM camptothecin for 25 min to cross-link topo I to DNA. Nuclei were prepared by hypotonic swelling of cells followed by NP40 treatment as described (*25*) and analyzed for non-DNA cross-linked topo I by SDS−PAGE/Western blot. To control for topo I recovery, NP40 nuclei prepared from 10^6 cells were DNase I-treated as described above, and solubilized material was analyzed for topo I by SDS-PAGE/Western blot.

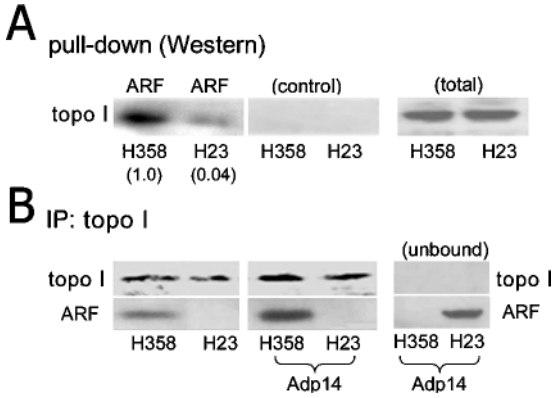

FIGURE 1: Analysis of ARF/topo I complexes in H358 and H23 cells. (A) Western analysis of topo I pulled down with immobilized full-length ARF fusion protein from H358 and H23 RIPA cell lysates (left panel) or nickel−NTA−agarose lacking fusion protein (control, middle panel). Right panel: Western analysis of total topo I protein in RIPA cell lysates of H358 and H23 cells. Each lane represents 200 μg of lysate protein. (B) Co-immunoprecipitation (IP) of topo I and ARF in H358 and H23 nuclear extracts before (left panel) or 48 h after treatment with Adp14, moi = 20 pfu/cell (middle panel). Two sequential co-IPs were pooled. Right panel shows a Western analysis of material that remained unbound following two sequential co-IPs. Each lane represents 175 μg of starting DNAse I-solubilized nuclear extract (about 10^6 cell equivalents).

RESULTS

Defective ARF/Topo I Complex Formation in H23 Lung Cancer Cells. An immobilized human ARF−thioredoxin fusion protein was used to pull down and compare ARF/topo I complexes in whole cell lysates prepared in RIPA to dissociate cellular and nuclear membranes. In H358 lung cancer cells (Figure 1A, left panel), topo I was present in the ARF fusion protein-bound fraction (see the Experimental Procedures), consistent with other published studies establishing topo I as an ARF binding protein (*8*, *9*). In H23 lung cancer cells, however (Figure 1A, left panel), we observed greatly reduced amounts of topo I in the ARF fusion protein-bound fraction. Digital analysis of band intensities (in parentheses below lanes) showed that bound topo I in H23 cells was about 4% that of H358 cells, and similar results were obtained in repeat assays. Control pull-down assays with nickel−NTA−agarose support alone did not pull down topo I from H358 or H23 cell lysates, confirming that binding was specific for ARF (Figure 1A, control). Endogenous topo I protein levels in RIPA lysates of H23 and H358 cells were similar, however (Figure 1A, right panel). Furthermore, a complete sequence analysis of the 2295 base pair coding sequence of topo I in H23 cells showed that the sequence corresponded to the wild-type topo I sequence (EC 5.99.1.2, Accession # NM_003286). Thus, reduced binding of topo I from H23 cells to immobilized ARF is neither the result of reduced cellular levels of topo I nor the result of a mutation in topo I that could alter its binding properties.

H23 cells also lacked endogenous ARF/topo I complexes. Figure 1B shows a co-immunoprecipitation experiment using

nuclear extracts solubilized by treatment with DNAse I, as described in the Experimental Procedures. This cellular fraction contains more than 95% of topo I and ARF relative to the cytoplasmic fraction (our unpublished observations and ref *20*). Extracts were immunoprecipitated with two sequential treatments with anti-topo I antibody, followed by SDS−PAGE/Western blot detection of ARF in the pooled immunoprecipitated material. Complexes were readily detectable in H358 nuclear extracts, but they were undetectable in H23 nuclear extracts (Figure 1B, left panel). A sequence analysis of ARF cDNAs from H358 and H23 cells confirmed that both were wild-type (see Experimental Procedures), indicating that an ARF defect did not account for the failure of the two proteins to form a complex. Because mutant and wild-type p53 have been reported to bind to topo I (*26*), we considered the possibility that competition between ARF and p53 for topo I binding might reduce ARF/topo I complexes in H23 cells, which express mutant p53 (*18*), but not in H358 cells, which are p53-null (*18*). However, we found no evidence for p53/topo I complexes in H23 cells, arguing against this possibility (data not shown). In addition, we carried out a control experiment in H358 cells before and after treatment with Adp53 (moi = 20), sufficient to induce expression of wild-type p53 at 48 h post-treatment to levels comparable to endogenous p53 levels in H23 cells, and found that recovery of ARF/topoisomerase I complexes was unchanged by this treatment (data not shown), consistent with results in H23.

The topoisomerase I protein we observe in the H23 and H358 cell lines migrates at a molecular weight of about 67 kDa, rather than the 91 kDa characteristic of the full length protein. A 67 kDa form has previously been observed in mammalian cells (*27*−*29*) and appears to be generated by proteolytic cleavage of the N-terminal domain of the larger form, leaving a fully active enzyme (*27*). Because we observe only the 67 kDa form following either direct lysis of whole cells for Western analysis (Figure 1A) or following DNAse I solubilization of nuclei (Figure 1B), and because complete protease inhibitors were present throughout both procedures, it is likely that this form represents a product generated in vivo. However, consistent with our observations in H23 cells, this form of topo I would not be expected to bind p53, since p53 appears to bind to the topo I N-terminal domain (*30*). The fact that this form binds to ARF, indicates that the N-terminal region of topo I is not required for complex formation with ARF.

Overexpressed ectopic ARF could not drive topo I into complexes with ARF in H23 cells, indicating that reduced ARF/topo I complex formation in H23 cells was neither a consequence of insufficient levels of endogenous ARF nor the unavailability of ARF. Co-immunoprecipitation/Western blot analysis showed that ARF/topo I complexes increase about 3-fold in H358 cells 48 h following a 4-h treatment with 20 pfu/cell of a replication-defective adenoviral vector encoding ARF (Adp14), as determined by digital analysis of band intensities, indicating that not all cellular topo I had been bound by ARF in untreated cells (Figure 1B, middle panel). Complexes were still undetectable in H23 cells following a similar treatment (Figure 1B, middle panel). The material that remained unbound following two successive immunoprecipitations with anti-topo I was also analyzed. Undetectable amounts of ARF protein in H358 cells were found in the unbound material, indicating that virtually all cellular ARF was complexed with topo I (Figure 1B, right-hand panel, unbound). In contrast, in H23 cells, ARF was detectable only in the unbound fraction, indicating that little or none of the cellular ARF complexed with topo I (Figure 1B, right-hand panel, unbound).

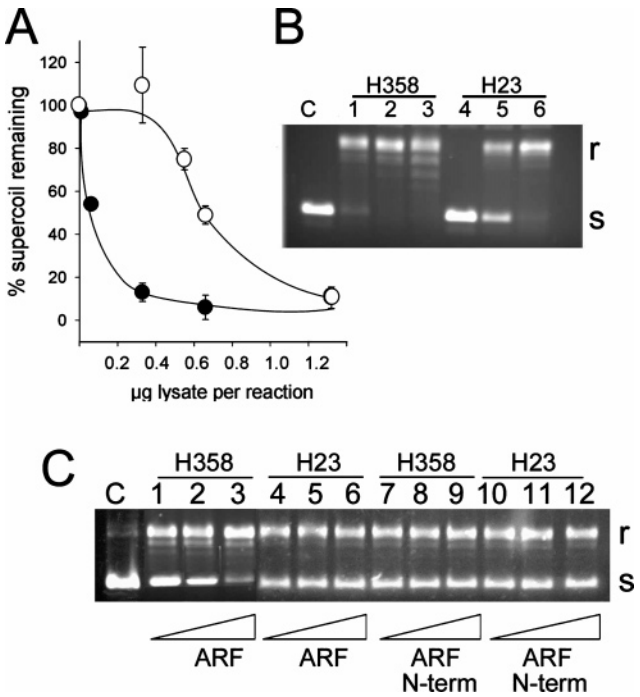

FIGURE 2: Comparison of topo I activity levels in H358 and H23 cells. (A) Graphical representation of in vitro topo I assay measuring loss of supercoiled plasmid DNA in the presence of increasing amounts of salt-extracted nuclear fractions from H358 (●) and H23 (○) cells (average of 2 independent assays). (B) Agarose gel electrophoresis of reaction products of a typical in vitro topo I assay in which 0.32, 0.65, or 1.3 μg of H358 extract (lanes 1−3, respectively) or H23 extract (lanes 4−6, respectively) were added per reaction. C = control, untreated supercoiled plasmid. (C) Agarose gel electrophoresis of reaction products of in vitro topo I assays of nuclear extracts of H358 (0.06 μg/reaction, lanes 1−3 and 7−9) and H23 (0.6 μg/reaction, lanes 4−6 and 10−12), carried out in the presence of increasing amounts (3, 9, 27 ng per reaction) of full-length ARF fusion protein (lanes 1−6) or ARF-N-terminal fusion protein (lanes 7−12). C = control, untreated plasmid, r = relaxed; s = supercoil.

Decreased Topo I Activity and Failure of ARF To Stimulate Topo I Activity from H23 Cells. Because ARF has been identified as an activator of topo I (*9*), we compared salt-extracted nuclear fractions of H23 and H358 cells for topo I activity in vitro and investigated how topo I activity was affected by the addition of increasing quantities of recombinant thioredoxin-ARF. As shown graphically in Figure 2A, we found that H358 topo I was more active at relaxing supercoiled plasmid DNA than was H23 topo I, achieving 50% relaxation at about 0.06 μg nuclear extract per reaction, some 10-fold lower than the amount of H23 extract needed to achieve the same level of relaxation (0.6 μg extract per reaction). Extracts from H23 and H358 cells were controlled by Western analysis and shown to contain equivalent levels of topo I (not shown). A typical electrophoretic profile of the topo I reaction products with increasing amounts of nuclear extract is shown in Figure 2B.

We then carried out similar assays using the amount of each respective nuclear extract that produced a 50% conver-

sion of supercoiled to relaxed form (0.06 and 0.6 μg extract protein per reaction for H358 and H23, respectively) and added increasing amounts of purified ARF fusion protein (3, 9, 27 ng). As a control, in separate assays, we added increasing amounts of thioredoxin-ARF-N-terminus, which does not bind to topo I (*8*). ARF fusion protein enhanced the activity of H358 topo I in a dose-dependent manner (Figure 2C, lanes 1−3), but had no effect on H23 topo I (Figure 2C, lanes 4−6), as expected on the basis of the inability of ARF to bind to H23 topo I (see previous section). Neither H358 nor H23 topo I activities responded to the addition of thioredoxin ARF-N-terminus at similar doses (Figure 2C, lanes 7−12).

Low Levels of Serine Phosphorylation in H23 Topo I Correlate with Low Activity and Failure To Bind ARF. Serine phosphorylation has been reported to activate topo I activity (*31*, *32*). A topo I immunoprecipitation analysis followed by Western detection of phosphoserine revealed that H358 cells expressed a serine-phosphorylated topo I (Figure 3A, lane 1, top row). A similar analysis of phosphotyrosine revealed no evidence for tyrosine phosphorylation (data not shown). Similar results were found in PC-3 cells (data not shown). In contrast, serine-phosphorylated topo I was only weakly detectable in H23 cells (Figure 3A, lane 2, top row). Treatment of both H358 and H23 nuclear extracts with alkaline phosphatase (AP) eliminated serine phosphorylation (Figure 3A, lanes 3, 4, top row) and abolished their topo I activity in vitro (Figure 3B, lanes 4−6 and 13−15). The dephosphorylated topo I from H358 cells could no longer be activated by addition of increasing amounts of ARF fusion protein (Figure 3B, lanes 7−9). Furthermore, while topo I co-immunoprecipitated with ARF from untreated H358 nuclear extracts (Figure 3A, lane 1, middle row), it failed to co-immunoprecipitate with ARF from H358 nuclear extracts treated with alkaline phosphatase (Figure 3A, lane 3, middle row). Topo I failed to co-immunoprecipitate with ARF from either untreated or alkaline phosphatase-treated H23 cell nuclear extracts (Figure 3A, lanes 2, 4, middle row). When alkaline phosphatase-treated extracts from either H358 or H23 cells were treated with casein kinase II (CKII), a serine kinase, we observed restoration of serine phosphorylation (Figure 3A, lanes 5, 6, top row) and restoration of ARF/topo I complex formation (Figure 3A, lanes 5, 6, middle row). Recovery of topo I following immunoprecipitation was the same in all cases (Figure 3A, lanes 1−6, bottom row).

Finally, we found that alkaline phosphatase treatment of purified recombinant human topoisomerase I abolished serine phosphorylation (Figure 3C, lane 1, top row) and abolished its ability to bind recombinant ARF fusion protein (Figure 3C, lane 1, middle row), but serine phosphorylation and ARF binding could be restored by treatment with casein kinase II (Figure 3C, lane 2, top and middle rows, respectively). Recovery of topo I following immunoprecipitation was the same in both cases (Figure 3C, lanes 1, 2 bottom row). Activity assays carried out on purified topo I following these same treatments showed that alkaline phosphatase treatment abolished activity and ARF-mediated activation of topo I (Figure 3D, lanes 2−4), but activity could be restored, together with ARF-mediated activation by treatment of the dephosphorylated topo I with casein kinase II (Figure 3D, lanes 5−7). The activities of untreated topo I ("un") and a

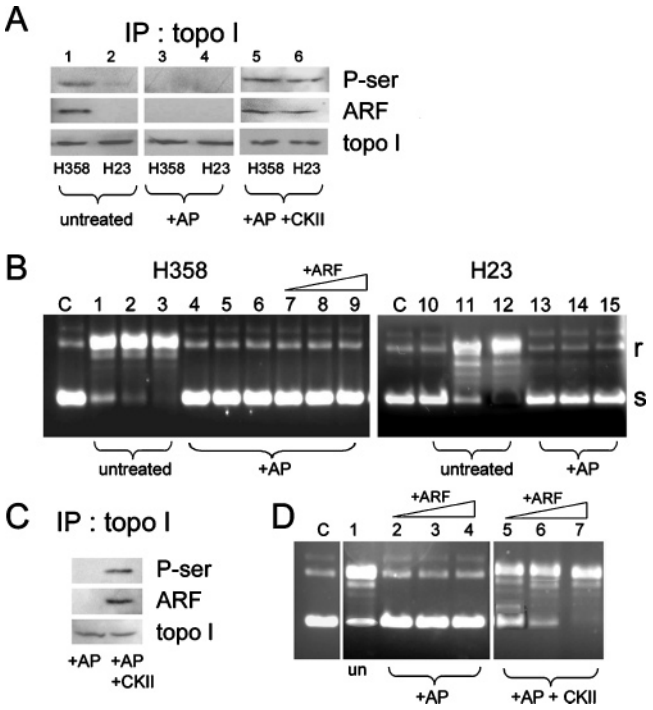

FIGURE 3: Relationship of topo I serine phosphorylation to topo I activity and ability to bind ARF. (A) Topo I immunoprecipitation (IP) followed by phosphoserine Western analysis (top row), ARF Western analysis (middle row) or topo I control Western analysis (lower row) of DNAse I-solubilized nuclear extracts of H358 and H23, before alkaline phosphate treatment (untreated, lanes 1, 2), after alkaline phosphatase treatment (+AP, lanes 2, 3), and after alkaline phosphatase treatment followed by treatment with casein kinase II (+AP +CKII, lanes 5, 6). Each lane represents 175 μg of starting DNAse I-solubilized nuclear extract. (B) Topo I assay using 0.1, 0.3, and 0.9 μg per reaction of H358 or H23 nuclear extracts before (lanes 1−3 and lanes 10−12, respectively) or after (lanes 4−9 and lanes 13−15, respectively) treatment with alkaline phosphatase (+AP). Lanes 7−9 show the topo I activity of 0.1 μg of alkaline phosphate-treated nuclear extract from H358 cells in the presence of 3, 9, and 27 ng of full-length ARF fusion protein, respectively. C = control, untreated plasmid; r = relaxed; s = supercoil. (C) Topo I immunoprecipitation/Western analyses as in part A, except that purified baculovirus-expressed human topo I (TopoGEN) and purified ARF fusion protein were used. Left lane (+AP) shows results with topo I treated with alkaline phosphatase. Right lane (+AP +CKII) shows results with topo I treated with alkaline phosphatase and then casein kinase II. Top row: Western analysis of serine phosphorylation. Middle row: Western analysis of ARF. Bottom row: Western analysis of topo I. Each lane represents a mixture of 10 pmol (1μg) of topo I and 10 pmol (0.14 μg) of ARF fusion protein. (D) Topo I assay using 0.5 unit of purified baculovirus-expressed human topo I (TopoGEN) treated as follows: lane 1, no treatment; lanes 2−4, alkaline phosphatase treatment, assayed in the presence of 3, 9, and 27 ng of ARF fusion protein, respectively; lanes 5−7, alkaline phosphatase treatment, followed by casein kinase II treatment, assayed in the presence of 3, 9, and 27 ng of ARF fusion protein, respectively. Lane "c" is control, i.e., plasmid only.

control reaction with plasmid only ("c") are shown in lanes 1 and c, respectively. The results establish that differences in topo I serine phosphorylation account for the differences in ARF/topo I complex formation in H358 and H23 cells, as well as for the observed differences in topo I activity.

Regulation of Topoisomerase I/DNA Binding by ARF. We carried out several topo I/DNA binding assays to address the mechanism by which ARF activates topo I. In Figure 4A we carried out an immunodepletion assay in which topo I was trapped in a complex with DNA by treatment of cells

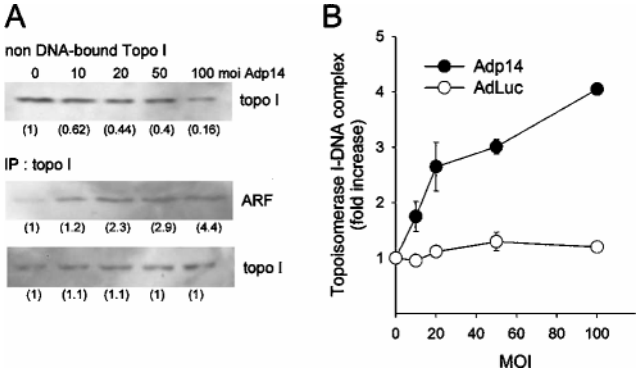

FIGURE 4: Regulation of topo I/DNA binding by ARF. (A) (top panel) Immunodepletion assay carried out on nuclei prepared from H358 cells treated with increasing doses of Adp14, followed by camptothecin to cross-link topo I onto DNA; (middle and bottom panels) topo I immunoprecipitation followed by ARF or topo I Western analysis. Digital analyses of topo I and ARF levels are shown below the lanes. (B) Co-precipitated topo I/DNA complexes present in K+$^-$SDS precipitated H358 cell lysates. Cells were treated with increasing doses of Adp14 or AdLuc, followed by labeling with [^{3}H]thymidine and treatment with camptothecin.

with camptothecin, followed by Western analysis of nuclei prepared with NP40 (see Experimental Procedures). Because topo I/DNA complexes are too large to enter the gel, an increase in topo I/DNA complex formation leads to a decrease in the intensity of the topo I immunoreactive band representing non-DNA-bound topo I. We found that treatment of H358 cells with increasing doses of Adp14 resulted in a progressive decrease in non-DNA-bound topo I (Figure 4A, top panel), under conditions where co-immunoprecipitated ARF/topo I complexes, released from NP40 nuclei by DNase I treatment, increased (Figure 4A, middle panel) and total topo I, released from NP40 nuclei by DNase I treatment, remained constant (Figure 4A, bottom panel). To further confirm that increased ARF/topo I complex formation leads to increased topo I/DNA binding, we carried out a second experiment in which vector-treated cells were labeled with [^{3}H]thymidine prior to treatment with camptothecin. Cellular protein was precipitated in the presence of potassium chloride and SDS, and the amount of coprecipitating DNA was determined by scintillation counting. As shown in Figure 4B, treatments with increasing doses of Adp14 but not AdLuc (control) led to a progressive increase in the amount of co-precipitating DNA, indicative of topo I/DNA complexes. Thus, increased ARF/topo I complex formation is accompanied by an increase in DNA-bound topo I.

ARF Enhances Cellular Sensitivity to Camptothecin-Induced DNA Damage Independent of p53. The fact that ARF enhanced the formation of cleavage complexes in the presence of camptothecin suggested that cellular responses to camptothecin and other topo I targeted therapies might be enhanced by ARF. We therefore used adenoviral vectors to achieve ectopic overexpression of full-length ARF (Adp14) or ARF-N-terminal domain (Ad1β) and used RNA interference to down-regulate endogenous expression of ARF. As shown by the Western analysis of H358 cells in Figure 5A, ARF levels increased by some 3-fold, as determined by digital analysis of band intensities, by 48 h post-treatment with Adp14 (moi, 20 pfu/cell, Figure 5A, lane 1), relative to Ad1β-treated cells (Figure 5A, lane 2) or untreated cells (Figure 5A, lane 4). By 72 h post-transfection of an siRNA expression plasmid to ARF exon 2 (Figure 5A, lane 5), endogenous ARF levels fell to 0.27 that found in untreated cells (Figure 5A, lane 4) or control siRNA-treated cells (Figure 5A, lane 3).

Viability assays were carried out 24 h after vector treatment by exposing cells for 24 h to increasing doses of camptothecin in triplicate in a 96-well viability assay and by assaying them for viability 5 days after the start of vector treatment (Figure 5B). For each growth curve, cell viabilities were normalized to the viability of cells treated with vector only (no camptothecin), to enable a direct visualization of the sensitization effect. As shown for H358 cells in Figure 5B (left assay), treatment of cells with Adp14 resulted in a greater decrease in cell viability with increasing camptothecin concentrations than did camptothecin alone. In contrast, treatment of cells with siRNA to reduce ARF expression resulted in a smaller decrease in cell viability with increasing camptothecin concentrations. Ad1β-treated cells overexpressing the ARF-N-terminal domain that does not interact with topo I, and control siRNA-treated cells in which levels of endogenous ARF remained unaltered displayed camptothecin responses similar to cells receiving no vector treatment (Figure 5B, left). Cells treated with control AdLuc vector displayed a sensitivity to camptothecin similar to untreated cells (not shown).

To verify the generality of these observations, the same series of assays were carried out with the PC-3 prostate cancer cell line (Figure 5B, right), with similar results. PC-3 cells express active, serine-phosphorylated topo I (data not shown). The siRNA used to down-regulate endogenous ARF targets the exon 2-encoded region of ARF that is shared by the p16INK4A tumor suppressor. While H358 cells express endogenous p16INK4A, PC-3 cells do not (*33*), and they therefore provide a control showing that the observed effect on camptothecin sensitivity can be attributed to ARF and is not cell specific. As a further siRNA control, we reduced endogenous ARF expression in H358 cells by treating them with an siRNA to exon 1β, which is not shared with p16INK4A, and then restored ARF expression by treatment with Adp14 1 day later. As shown in the Western analysis in Figure 5C, siRNA treatment (lane 3, ARF) reduced ARF protein levels to about 0.25 that of untreated cells (lane 1, ARF) by 72 h post-siRNA treatment. Digital analyses of ARF band intensities are shown below the ARF lanes. Treatment with Adp14 (moi, 100 pfu/cell) 24 h after siRNA treatment restored ARF expression, measured 72 h after siRNA treatment, to 1.3-fold that found in untreated cells (lane 2, ARF). Actin levels remained unchanged by these treatments (Figure 5C, actin). To assay how these treatments affected camptothecin responses, non-vector-treated cells, siRNA-treated cells, and siRNA+Adp14-treated cells were exposed to increasing doses of camptothecin as in Figure 5B and assayed for viability 5 days after the start of vector treatment. As shown by the viability assay in Figure 5C, reduction in ARF expression in H358 cells following exon 1β siRNA treatment resulted in decreased sensitivity to camptothecin, while restoration and moderate overexpression of ectopic ARF slightly enhanced sensitivity, supporting the results in Figure 5B.

We found that the increased camptothecin sensitivity of Adp14-treated H358 cells correlated with about a 3-fold increase in ARF/topo I complex formation relative to Ad1β-

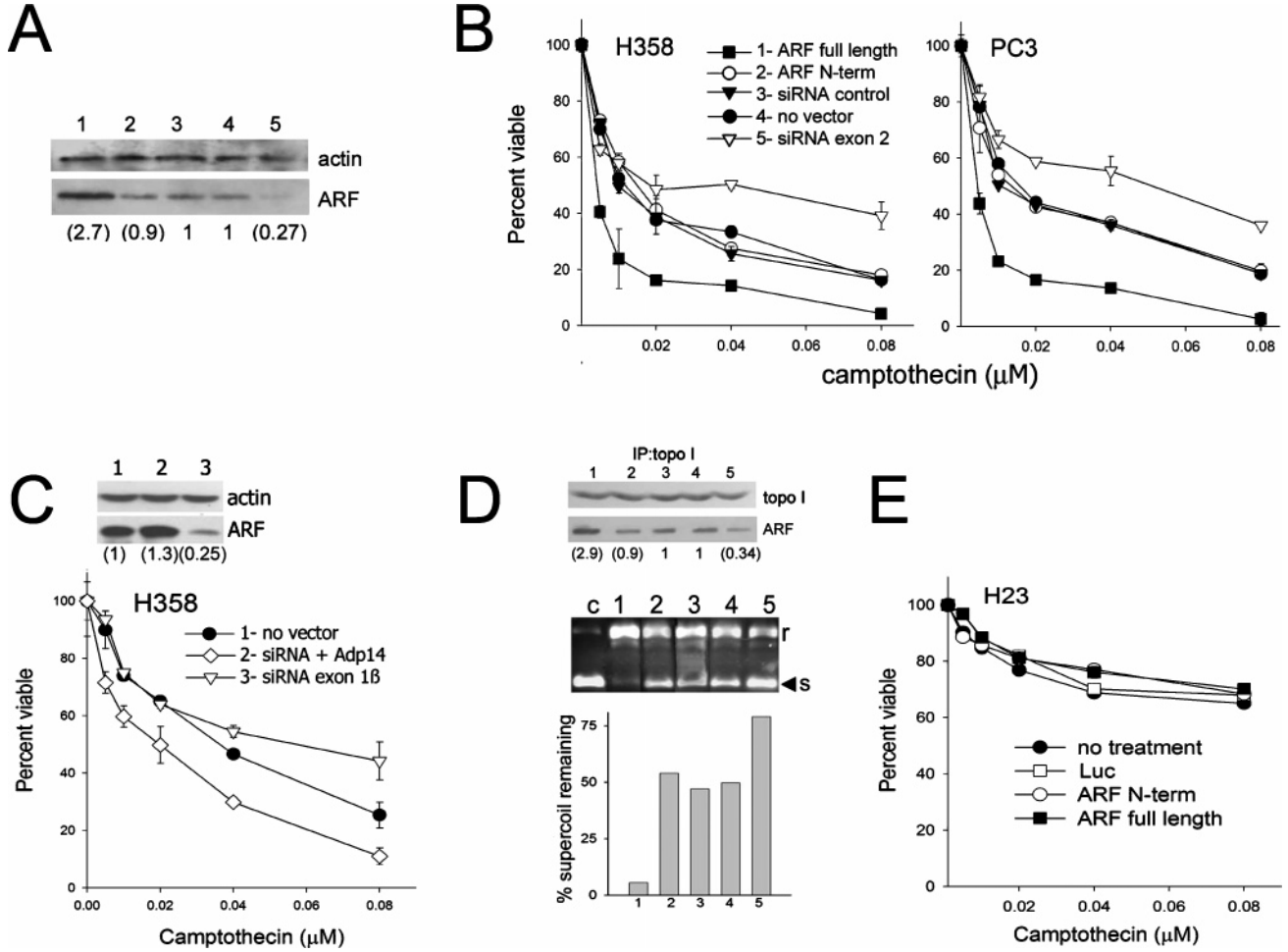

FIGURE 5: Regulation of camptothecin sensitivity by the ARF/topo I complex. (A) Western analysis of H358 cellular actin (top row) or ARF (bottom row) 48 h after treatment with Adp14 (lane 1) or Ad1β (lane 2) or 72 h after treatment with siRNA control sequence (lane 3) or ARF siRNA to exon 2 (lane 5). Lane 4 shows actin and ARF levels in untreated H358 cells. Digital analyses of ARF band intensities are shown beneath the ARF Western blot. (B) H358 and PC-3 cell viabilities assayed 5 days after vector treatment (adenoviral vector, moi 20 pfu/cell, or siRNA) and 4 days post-treatment with increasing doses of camptothecin. Viability is expressed as a percent of no-camptothecin control for each vector or siRNA treatment. Results represent the average of triplicate wells, with standard deviations indicated. Treatments: Adp14 (ARF full length, ■), Ad1β (ARF N-term, ○), siRNA control (▼), no vector (●), siRNA exon 2 (▽). (C) Western analysis (top panel) of H23 cellular actin and ARF levels in untreated cells (lane 1) or 72 h after treatment with ARF siRNA to exon 1β (lane 3) or siRNA plus Adp14 (moi = 100) (lane 2). Digital analyses of ARF levels are shown below ARF lanes. (Lower panel) H358 cell viability assay carried out as in part A following the indicated treatments. Viability was measured 3 days after the start of camptothecin treatment. (D) (Upper panel) Topo I immunoprecipitation followed by topo I or ARF Western analysis in H358 cells. Lanes correspond to the same treatments as in A. Digital analyses of ARF band intensities are shown below the ARF lanes. (Lower panel) Ethidium bromide-stained agarose gel of the reaction products of an in vitro topo I assay measuring loss of supercoiled plasmid DNA in the presence of 0.75 μg of H358 nuclear extract (amount that converts 50% of supercoiled plasmid to the relaxed form; see Figure 2A). Numbered lanes correspond to the same treatments as in A. Far left lane ("c") shows plasmid only (no extract); s = supercoiled; r = relaxed form. A graphical representation of the relative supercoil band intensities of lanes 1−5 of the ethidium bromide-stained agarose gel, determined by digital analysis and expressed as a percentage of the supercoil band intensity in the plasmid-only control lane "c", is shown in the bar graph below the agarose gel. (E) H23 cell viability assay carried as in part A, following the treatments described in part B or with control vector, AdLuc (Luc □).

treated, control siRNA-treated, or non-vector-treated cells, as shown by the IP/Western analysis in Figure 5D (upper panel, lane 1 versus lanes 2−4), and with an increase in topo I activity (Figure 5D, lower panel lane 1, bar 1 versus lanes 2−4, bars 2−4). The decreased camptothecin sensitivity of siRNA-treated H358 cells correlated with about a 3-fold decrease in ARF/topo I complex formation (Figure 5D, upper panel, lane 5) and with a decrease in topo I activity (Figure 5D, lower panel, lane 5, bar 5).

The H23 cell line, with low to undetectable levels of endogenous ARF/topo I complexes, respectively (Figure 1B) displayed a greatly reduced response to camptothecin (Figure 5E), consistent with studies showing that loss of topo I phosphorylation reduces activity (*32*). The fact that H23 cells cannot be sensitized to camptothecin by ectopic overexpression of ARF indicates that ARF-mediated sensitization requires its interaction with active, serine phosphorylated topo I. We repeated the H23 and H358 cell viability assays with Camptosar (Ironotecan, camptothecin-11), a camptothecin-related chemotherapeutic drug that acts through the same mechanism as camptothecin (*34*). We observed a similar pattern of resistance and sensitivity, respectively, for H23 and H358 (data not shown). We also observed ARF-mediated sensitization to Camptosar for H358 but not H23, indicating that the results observed for camptothecin extend to camptothecin-like chemotherapeutic agents as well (data not shown).

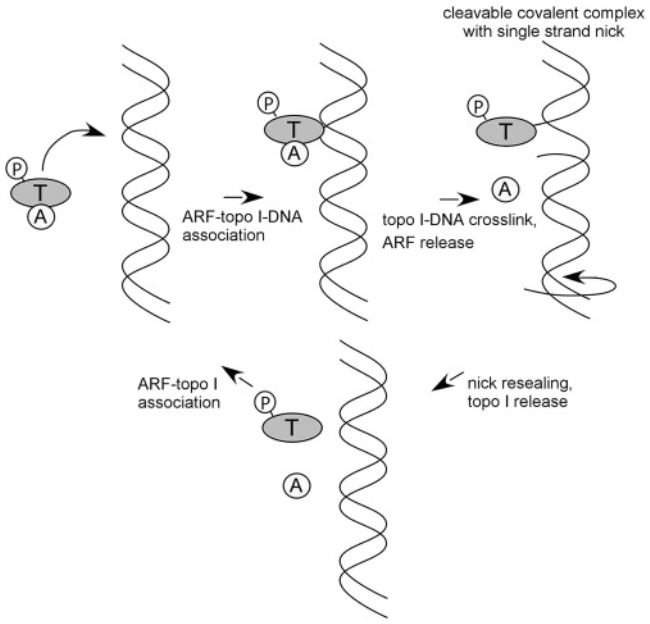

FIGURE 6: Model of ARF-mediated activation of topo I. Model shows ARF binding to and promoting topo I association with DNA. The initiation of the enzymatic reaction leading to topo I covalent binding to DNA and DNA single-strand nicking results in release of ARF. Topo I is released following unwinding and resealing the nick. A = ARF; T = topo I, P = phosphoserine.

DISCUSSION

This study has shown that the ARF tumor suppressor coregulates topo I through a serine phosphorylation-dependent interaction with topo I that promotes increased association of topo I with DNA and leads to enhanced DNA cleavage in the presence of camptothecin. The interaction does not appear to involve the topo I N-terminal domain. Topo I is known to be a phosphoprotein (*32*) that copurifies with serine kinase activities that resemble casein kinase II (CKII) (*35*) and protein kinase C (PKC) (*32*). Phosphorylation occurs primarily on serine residues in vivo (*23*, *35*−*37*) and appears to be necessary for the initial complex formation between topo I and DNA (*36*). We find that the activity of topo I and its ability to complex with ARF is abolished by treatment with alkaline phosphatase and that activity and complex formation with ARF is restored by treatment with CKII. The activity of unphosphorylated topo I has also been reported to be restored by treatment with PKC (*32*, *37*). However, a comparison of in vivo and in vitro phosphopeptide maps has suggested that casein kinase II is the primary phosphorylating enzyme in vivo (*38*, *39*). The c-abl tyrosine kinase has also been reported to activate topo I (*40*), and this activity may play a role in certain situations. However, we find no evidence for tyrosine phosphorylation on topo I in the H358, H23, and PC-3 cell lines used in this study, arguing against a role for the c-abl kinase in these cells.

Our results suggest a model in which ARF enhances cellular sensitivity to camptothecin by interacting with serine phosphorylated topo I and promoting its recruitment to DNA, as depicted by the scheme in Figure 6. The increased level of DNA-bound topo I that results from ectopic overexpression of ARF would lead to increased single-strand DNA nicking in the presence of camptothecin and enhanced cellular sensitivity to camptothecin. Although we detect ARF/topo I complexes following the 25 min exposure to camptothecin used to cross-link topo I to DNA, ARF appears to slowly dissociate from topo I in the presence of camptothecin, as we do not detect ARF/topo I complexes after 3 h of continuous camptothecin treatment (Bandyopadhyay, unpublished). This suggests that the formation of the covalent complex may destabilize the ARF/topo I interaction, as depicted in the model. Following unwinding of the DNA, the single-strand nick is resealed, with the release of topo I, and the cycle can repeat. Given the complexity of the chromatin substrate for topo I and the fact that both ARF and topo I are known to interact with a variety of other proteins (*5*, *13*), it is likely that the scheme in Figure 6 will involve additional regulatory features that remain to be elucidated. In particular, topo I is known to interact with a large array of proteins besides p53 and ARF that could also be important to topo I function and affect cellular sensitivity to camptothecin in H23 cells. These include casein kinase II (*35*), HMG proteins (*41*), PARP-1 (*42*), RNA-splicing factor PSF/p54nrb (*43*), Werner's syndrome protein (*44*), nucleolin (*45*), RNA pol II (*46*), TATA binding protein (*47*), and topors (topo I-binding Arg-Ser protein) (*48*). Recently, a proteomic analysis of topo I binding proteins from Hela cell extracts revealed some 29 additional nuclear proteins that bind to topo I (*49*). Most of the topo I interactions that have been identified involve the N-terminal domain of topo I, with certain proteins, most likely including ARF, interacting with the core domain (*49*, *50*).

The mechanisms that regulate the extent of camptothecin-induced DNA cleavage are of considerable clinical interest, since they impact the success of camptothecin-based chemotherapeutic regimens (*11*, *51*). An important conclusion from this study is that the cellular response to camptothecin is enhanced by ectopic expression of ARF in proportion to the increased cellular levels of the ARF/topo I complex. Furthermore, the effect is independent of ARF's p53-dependent activity, since the cell lines we have used lack endogenous wild-type p53 activity. Consistent with our observation that the H23 cell line expresses an underphosphorylated topo I that binds poorly to ARF is our finding that the cellular response to camptothecin is unaffected by ectopic overexpression of ARF in this cell line.

Various mechanisms contribute to cellular resistance to camptothecin-like topo I poisons, including decreased intracellular drug accumulation (*15*), decreased expression of topo I (*52*), or mutation of topo I (*53*−*56*). Lowered phosphorylation of topo I due to the defective action of CKII in a murine lymphoma cell line has also been shown to confer camptothecin resistance (*57*), and c-abl tyrosine kinase-mediated phosphorylation of topo I has been shown to activate topo I and confer camptothecin sensitivity (*40*). The results of the present study indicate that ARF-mediated coactivation of serine phosphorylated topo I further increases camptothecin sensitivity and suggest that cancer-associated abnormalities in topo I that impair its ability to interact with ARF could be indicative of cancer cells with resistance to camptothecin. In cancer cells that retain the ability to form ARF/topo I complexes, ectopic overexpression of ARF, or a portion of ARF corresponding to the topo I interacting region, could therefore be used clinically to enhance tumor responses to camptothecin-like chemotherapies. Our in vitro assays with H358 cells in Figure 5 show that moderate overexpression of ARF (about 3-fold higher than endogenous

levels) results in a 3−4-fold increase in sensitivity to camptothecin in vitro. Although it is not clear that such a difference will be clinically relevant, it is important to note that levels of in vitro cisplatin resistance of ovarian cancer cells as small as 2.5-fold have translated into clearly reduced responses in vivo in mouse xenograft models (*58*, *59*), suggesting that even small differences in sensitivity could potentially have clinical relevance. Further studies will be needed, however, to validate this.

The fact that most cellular ARF is present as a chromatin-bound nucleolar protein that associates with the rRNA promoter (*20*) has suggested a role in cellular regulation beyond its involvement with the p53 pathway, an activity that does not involve DNA binding and that occurs in the nucleoplasm. Several studies have suggested a role for ARF in regulating the activity of nucleophosmin (NPM/B23) (*60*), an abundant nucleolar protein involved in the maturation of 28S ribosomal RNA (*61*). ARF has been shown to promote NPM/B23 degradation (*62*) and inhibit ribosomal RNA processing (*63*). ARF has also been shown to inhibit ribosomal RNA transcription (*64*). The fact that ARF interacts with topo I, a nucleolar chromatin-bound enzyme required for DNA replication and for transcription of ribosomal RNA, and the fact that ARF stimulates topo I activity suggest a role in regulating a topo I-associated activity in transcription or replication. ARF's nucleolar and nucleoplasmic locations appear to represent distinct activities of ARF during cell growth or stress-induced growth arrest, respectively, and involve distinct ARF interactions (*5*, *21*, *65*, *66*). Further studies of the biological regulation of the ARF/topo I complex could therefore provide additional insight into the nucleolar and nucleoplasmic activities of ARF and the broader roles of both proteins in cellular regulation and in cancer.

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We dedicate this work to the memory of Prof. Pierre Potier.

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