Purification of Topoisomerase II from Amsacrine-resistant P388 Leukemia Cells

EVIDENCE FOR TWO FORMS OF THE EnZYME*

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Topoisomerase II was purified from an amsacrine-resistant mutant of P388 leukemia. A procedure has been developed which allows the rapid purification of nearly homogeneous enzyme in quantities sufficient for enzyme studies or production of specific antisera. The purified topoisomerase II migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as two bands with apparent molecular masses of 180 (p180) and 170 kDa (p170); both proteins unnotted P4 DNA in an ATP-dependent manner and displayed amsacrine-stimulated covalent attachment to DNA. Staphylococcus V8 protease cleavage patterns of p170 and p180 showed distinct differences. Specific polyclonal antibodies to either p170 or p180 recognized very selectively the form of the enzyme used to generate the antibodies. Immunoblotting with these specific antibodies showed that both p180 and p170 were present in cells lysed immediately in boiling sodium dodecyl sulfate. Comparison of the purified topoisomerase II from amsacrine-resistant P388 with that from amsacrine-sensitive P388 demonstrated that each cell type contained both p180 and p170; however, the relative amounts of the two proteins were consistently different in the two cell types. The data strongly suggest that p170 is not a proteolytic fragment of p180. Thus, P388 cells appear to contain two distinct forms of topoisomerase II.

Topoisomerases are enzymes which control the topological state of DNA (for reviews, see Refs. 1–3). Type II topoisomerases catalyze DNA strand passage through transient double strand breaks in the DNA; this ability to change the linking number of DNA allows the enzymes to mediate DNA interconversions such as supercoiling (demonstrated for prokaryotic only) and relaxation of supercoiling, catenation and conversions such as supercoiling (demonstrated for prokaryotic only) and relaxation of supercoiling, catenation and decatenation, and knotting and unknotting (1–3). Topoisomerase II appears to be a component of the nuclear scaffold (4, 5), perhaps located near the base of chromatin loops (6, 7). Indirect evidence suggests that the enzyme may be associated with gene enhancer regions (8). These enzymes have been implicated in a number of critical cellular processes including DNA replication and transcription (1–3) and chromosomal segregation (9–11).

An important feature of the topoisomerase II-mediated DNA strand passage reaction is the covalent attachment of the enzyme to the DNA via a tyrosine-DNA phosphodiester linkage; under denaturing conditions the enzyme can be trapped in this form, resulting in topoisomerase-associated DNA strand breakage (12). Of particular interest is the observation that several structurally diverse classes of antitumor agents such as anthracyclines, anthracenediones, epipodophyllotoxins, and ellipticines greatly increase the number of topoisomerase II-associated strand breaks in DNA (13–15). This has led to the suggestion that topoisomerase II is a common target of these drugs (14). Consistent with this idea has been the finding that several cell lines which have developed resistance to one class of topoisomerase II-active compounds are cross-resistant to other topoisomerase II-active compounds (16–19). For example, a murine leukemia cell line which has developed resistance to amsacrine (P388/A20) is cross-resistant to teniposide, bisantrene, and doxorubicin, but not to the topoisomerase I-active drug, camptothecin; resistance to these drugs could not be explained by differential uptake (20). Furthermore, the amsacrine-resistant cell line contained 2–3-fold less topoisomerase II activity than its parental line, as well as reduced topoisomerase II immunoreactivity (20). These data suggest that resistance to amsacrine in P388/A20 may be due to changes in the expression or chemosensitivity of topoisomerase II.

To test the hypothesis that resistance to amsacrine in P388/A20 is due to an alteration in topoisomerase II and to permit detailed study of the enzyme, topoisomerase II has been purified from P388/A20 cells. A purification scheme is described which is both rapid and reproducible and provides quantities of highly purified topoisomerase II sufficient for enzyme studies and development of topoisomerase-specific antisera. Topoisomerase II from amsacrine-resistant P388 cells was purified as two distinct polypeptides of 180 and 170 kDa. Both polypeptides had topoisomerase II activity. Differences in the antigenicity and proteolytic cleavage patterns of the two forms of the enzyme strongly suggest that the 170-kDa enzyme is not simply a proteolytic fragment of the 180-kDa enzyme.
Two Forms of Topoisomerase II

kDa enzyme. Furthermore, both the 180- and 170-kDa forms of topoisomerase II are also found in amuscsensitive P388 cells, though the ratio of the two bands is consistently different from that found in P388/A20 cells.

EXPERIMENTAL PROCEDURES

**Materials** - Leupeptin, PMSF, benzamidine, soybean trypsin inhibitor, 2-mercaptoethanol, and Tris were from Sigma. Glycerol and potassium phosphate were from J. T. Baker Chemical Co. Ultrogel hydroxylapatite was from LKB; Mono Q, Mono S, and Fast Flow Q were from Pharmacia Biotechnology, Inc. All electrophoresis chemicals were from Bio-Rad. Molecular weight standards were from Bio-Rad (high molecular weight kit) or Sigma (prestained kit). HEPES was from Research Organics (Cleveland). Freund's complete and incomplete adjuvants were from GIBCO. AuroProbe BL GAR, AuroDye, and IntensSE were from Janssen. Staphylococcus V8 protease was from Worthington. Nicotinucleoside (0.2 mM pere) was from Schleicher and Schuell. CHAPS and IODO-GEN were obtained from Pierce Chemical Co.

**Cells** - P388/A20 was cloned in vitro from P388/amsacrine by growth in soft agar (20, 21). P388/WT20 was similarly cloned from P388/S cells (20, 21). Cells were grown intraperitoneally in B6D2F mice as described previously (21) and harvested while in exponential growth.

**Purification of Topoisomerase II** - All steps were performed at 0-4°C. Chromatography was done on a Pharmacia Biotechnology, Inc. fast protein liquid chromatography system composed of two P-500 pumps and an LCC-500 controller.

**Topoisomerase II Assay** - On day 7 postinoculation, the ascites tumors were removed from syngeneic mice and placed on ice. The cells were washed three times in 4-5 volumes of PBS containing 1 mM PMSF, 1 mM benzamide, 1 µg/ml soybean trypsin inhibitor and centrifuged for 5 min at 1000 × g in a Beckman J-6M centrifuge. The cells were resuspended in 4 volumes (3.5-2.0 × 10^9 cells/ml) of 5 mM potassium phosphate, pH 7.0, 2 mM MgCl2, 0.1 mM EDTA, 1 mM PMSF, 1 mM benzamide, 10 µg/ml soybean trypsin inhibitor, 50 µg/ml leupeptin, 10 µM 2-mercaptoethanol and stirred slowly with a magnetic stirring bar at 4°C for 15 min. Nuclei were prepared by lysis of the cells with 10 strokes from a chilled Dounce homogenizer. The extent of cell lysis was monitored by microscopy. The lysate was centrifuged for 10 min at 1000 × g. The nuclei were washed twice with 5 volumes of 1 mM potassium phosphate, pH 6.5, 5 mM MgCl2, 1 mM EGTA, 10% glycerol, 100 mM NaCl, 1 mM PMSF, 1 mM benzamide, 10 µg/ml soybean trypsin inhibitor, 50 µg/ml leupeptin, and 10 mM 2-mercaptoethanol by centrifuging at 1000 × g for 5 min. The nuclei were resuspended in 5 volumes of reaction buffer (5 mM potassium phosphate, pH 7.0, 2 mM MgCl2, 0.1 mM EDTA, 1 mM PMSF, 1 mM benzamide, 10 µg/ml soybean trypsin inhibitor, 50 µg/ml leupeptin, 10 mM 2-mercaptoethanol, and stirred slowly with a magnetic stirring bar at 4°C for 15 min. Nuclei were prepared by lysis of the cells with 10 strokes from a chilled Dounce homogenizer.

**Antibodies** - Purified topoisomerase II was electrophoresed on SDS-polyacrylamide gels to separate the 180-kDa band (from 170-kDa band (approximately 25 µg of each band). Each gel was washed with the appropriate amount of gradually diluted with Q buffer at 0.5 ml/min. One-milliliter fractions were collected (0.5 ml). Fractions were assayed for P4 unknotting activity; active fractions were pooled for Mono S chromatography.

**Mono S** - A Mono S column (0.5 × 5 cm) was washed extensively according to manufacturer's instructions. Four gradients of 0-1 M NaCl in S buffer (0.5 mM EDTA, 50% glycerol) were run, and the column was then equilibrated with 0.2 M NaCl in S buffer. The pooled fractions from Mono Q or Fast Flow Q were diluted with an equal volume of S buffer and loaded onto the column at 1 ml/min. After the column was washed with 5-10 volumes of 0.2 µM NaCl in Q buffer, topoisomerase II was eluted with a 30-40% gradient of 0.2-0.6 M NaCl in S buffer at 0.5 ml/min. Fractions (0.5 ml) were assayed for P4 unknotting activity; active fractions were pooled with an equal volume of glycerol and stored at -20°C.

**Topoisomerase II Antibodies** - Pured topoisomerase II was electrophoresed on SDS-polyacrylamide gels to separate the 180-kDa band from the 170-kDa band (approximately 25 µg of each band). After staining of the gel with Coomassie Blue, the individual bands were excised from the gel and placed into PBS. The gel slices were crushed with a Dounce homogenizer, an equal volume of Freund's complete adjuvant was added, and the samples were emulsified by brief sonication. New Zealand White rabbits were injected intradermally or subcutaneously with the antigen. Booster injections (approximately 10 µg/band) were given every 3-5 weeks and every 3-4 weeks thereafter. Animals were bled from the central ear artery 7-13 days after each boost. Sera were stored at -70°C.

**Western Transfer and Immunoblotting** - Following SDS-PAGE, gels were electrophoretically transferred (Hoeffer Transfer, TE 52) to nitrocellulose at 400 mA for 4-6 h (26). Blots were blocked with 0.5% gelatin in PBS and probed with anti-topoisomerase II antibodies appropriately diluted into WBWB (25 mM Tris-HCl, pH 7.5, 1% CHAPS, 10 mM 2-mercaptoethanol). P4 DNA (0.2 µg/assay) was diluted into 2× reaction buffer (100 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM MgCl2, 1 mM EDTA, 80 µg/ml bovine serum albumin) containing 2 mM ATP. Five minutes later, the solution was incubated at 37°C for 5 µl of stop solution (5% SDS, 50 mM EDTA, 50% glycerol, 0.5 mg/ml bromophenol blue). The reaction mixture was electrophoresed on a 0.7% agarose gel in TPE buffer (80 mM Tris, 2 mM EDTA); the gel was then stained with ethidium bromide and photographed with Polaroid Type 55 film. Photograph negatives were scanned with a Beckman DU-SB spectrophotometer to quantify the amount of unknotted P4 DNA (23).

**SDS-PAGE** - Samples were electrophoresed on a Bio-Rad Protein II (160 × 160 × 0.75 mm) according to the method of Laemmli (24). Transfers (67% runs) were run at 5 mA/gel for 14-16 h and stained with silver according to the method of Merrill et al. (25).

**Inhibition of P4 Unknotting Activity by Anti-pi70 Antibody** - Anti-pi70 serum or preimmune serum was precipitated with 50% ammonium sulfate; the IgG precipitate was redissolved in PBS and desalted over G-50. Fractions from Mono S containing predominantly pi70 or pi80 were first diluted into 10 mM Tris-HCl, pH 7.5, 1% CHAPS, 10 mM 2-mercaptoethanol and then added to an equal volume of either 0.2 M NaCl in Q buffer at 0.5 ml/min. One-milliliter fractions were collected (0.5 ml). Fractions were assayed for P4 unknotting activity as described above.

Affinity Purification of Antibody - For affinity purification of an-
tibodies, the serum was first precipitated with 50% ammonium sulfate and desalted over G-50. The IgG fraction was diluted in WBWB and used to probe a nitrocellulose blot of purified topoisomerase II as described above. The topoisomerase band was cut from the nitrocellulose sheet, and the antibodies were extracted as described by Smith and Fisher (28). The extracted antibodies were diluted in WBWB/1% normal goat serum and stored at 4°C with 0.025% sodium azide.

Covalent Transfer of 32P from DNA to Protein—Covalent transfer of 32P from DNA to p170 and p180 was performed according to Rowe et al. (29, 30). Briefly, a 3.1-kilobase BamHI fragment of plasmid pGFEca (generously provided by Dr. Maxwell Lee, Duke University) was labeled with [32P]dCTP by the random priming technique (Prime Time, IBI Technologies). To this labeled DNA (2.5 ng) was added 1 μg of purified topoisomerase II (Mono S fractions pooled to contain approximately equal amounts of p170 and p180) in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 1 mM ATP, 30 Ba131 nuclease (Bethesda Research Laboratories) for 60 min at 30°C. The sample was then boiled for 10 min in an equal volume of SDS-sample buffer and electrophoresed as described above. Following silver staining, the gel was dried and exposed to Kodak XAR-5 film for 18 h with an intensifying screen.

Proteolytic Fragmentation of Topoisomerase II—Comparison of the proteolytic cleavage patterns of the 180- and 170-kDa proteins was done by the method of Cleveland et al. (31). Briefly, either purified topoisomerase II or radioiodinated purified topoisomerase II was electrophoresed on a 7% SDS-polyacrylamide gel and the bands of interest located by Coomassie Blue staining or autoradiography of the wet gel. The bands were carefully excised from the gel and loaded into the wells of a 7-25% gradient SDS-polyacrylamide gel. The gel slices were overlaid with 20% glycerol in Cleveland buffer (31), followed by the appropriate amount of Staphylococcus V8 protease in 10% glycerol in Cleveland buffer. The samples were electrophoresed at 6 mA/gel for 14-16 h and either stained with silver (25) or dried and exposed to Kodak XAR-5 film for 2-5 days at -70°C with an intensifying screen.

Radioiodination of Topoisomerase II (approximately 10 μg) was radioiodinated with 100 μCi of Na131I by 100 μg of IODO-GEN (32) dried onto 12 × 75-mm glass tubes. The topoisomerase II was diluted to 100 μl in 10 mM Tris-HCl, pH 7.5, 0.2% SDS. Free 131I was desalted over G-50 in microcentrifuge tubes (33).

Protein Determination—Protein concentrations were measured by the method of Bradford (34) with lysozyme as a standard.

RESULTS

The first step in the purification of topoisomerase II was a selective extraction of the enzyme from isolated P388/A20 nuclei with 0.35 M NaCl (35). This procedure extracts approximately 90% of the topoisomerase II from the isolated nuclei as judged either by the ability to re-extract topoisomerase II with more harsh conditions (e.g. 1 M NaCl or 1% CHAPS) or by immunoblot analysis of the extracted nuclei (data not shown). Selective extraction with 0.35 M NaCl also removed about 80% of the total cellular protein (Table I).

Protease inhibitors were included in each step of the nuclear isolation and 0.35 M NaCl extraction; each inhibitor was freshly prepared and added to the buffers just prior to use.

The 0.35 M NaCl extract was immediately loaded onto a column of Ultrogel hydroxyapatite which had been equilibrated with 0.2 M potassium phosphate. Greater than 90% of the protein which was loaded onto the column either passed through without binding or was eluted with a 0.2 M potassium phosphate wash (data not shown). Bound protein was eluted from the column with a gradient of 0.2-1.0 M potassium phosphate; topoisomerase II activity began to elute at 0.55-0.6 M potassium phosphate and formed a broad trailing peak. About 60% of the topoisomerase II activity loaded onto the hydroxyapatite column was recovered (Table I).

Active fractions from hydroxyapatite were pooled and applied to either Mono Q or Fast Flow Q. Mono Q gave the best separation of topoisomerase II but had a greater tendency to build up pressure during sample loading. Because of the high ionic strength of the sample after hydroxyapatite, the potassium phosphate concentration had to be reduced before loading. This was done by diluting the sample in 20 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM 2-mercaptoethanol (Q buffer) immediately before loading. Topoisomerase II activity eluted as a single peak at about 0.4 M NaCl and was associated with the appearance of two bands on SDS-PAGE at 180 (p180) and 170 kDa (p170) (Fig. 1, panels A–C). Recovery of topoisomerase II activity from Mono Q or Fast Flow Q was usually 25-50% but occasionally dropped below 10% (Table I). Sudden losses of topoisomerase II activity at the Q column step occurred more frequently when P388/A20 was used as the source of the enzyme than when P388/WT20 was used (data not shown). This likely represents enzyme denaturation (e.g. due to enzyme dilution) and may reflect a greater lability of the enzyme from the amssacrine-resistant cells. However, it may also reflect loss of the enzyme or perhaps even removal of an unknown stimulatory factor.

The final step in the purification was chromatography of the pooled Q fractions over Mono S, which separated the remaining proteins to give a nearly homogeneous preparation (Fig. 2A). Topoisomerase II binds to both Mono Q (anion exchange) and Mono S (cation exchange) at pH 7.5, requiring about 400 mM NaCl for elution from either column; proteins which co-purified with the enzyme over Q showed different elution profiles on Mono S. Depending upon the Q fractions that were pooled, minor contaminating proteins were sometimes observed in the purified preparations (e.g. Fig. 2A, 116 kDa). Comparison of Figs. 1B and 2A shows that these minor bands did not co-purify with topoisomerase II activity through all chromatographic steps but rather resulted from slight overlap of their peaks. Purification of the enzyme was about

| Table I
| Summary of purification |
|-------------------------|------------------------|
| Step                    | Volume | Total P4 unknotting activity | Total protein | Specific P4 unknotting activity | Recovery | Purification |
| A20 cells               |        | mg             |                | mg                      |         | %          |
| 0.35 M NaCl extract     |        | 20°            | 2.72 × 10⁴    | 4212                    | 6.46 × 10⁴ | 100        | 1          |
| Hydroxyapatite pool     |        | 150            | 2.45 × 10⁵    | 726                     | 3.37 × 10⁴ | 90°        | 5.2        |
| Fast Flow Q pool (Mono S load) | 47     | 1.86 × 10⁷ | 66.7          | 2.78 × 10⁵              | 68.8     | 43.0       |
| Mono S pool            |        | 2.2            | 1.11 × 10⁶    | 0.57                    | 1.95 × 10⁶ | 4.1        | 301.9      |
|                        |        | 6.0            | 5.83 × 10⁵    | 0.186                   | 3.17 × 10⁴ | 2.1        | 490.7      |

* Packed cell volume.
* Assuming 90% extraction.
Two Forms of Topoisomerase II

FIG. 1. Chromatography of topoisomerase II over fast flow Q. Pooled fractions from Ultrogel hydroxylapatite were chromatographed over Fast Flow Q as described under "Experimental Procedures." In each panel, the peak of topoisomerase II activity is indicated by the arrow. Panel A, column elution profile. The solid line indicates absorbance at 280 nm; the dashed line is the NaCl gradient. Panel B, the total protein pattern of the fractions from the peak of topoisomerase II activity is shown. An equal volume of each fraction (1-2 µg of protein/lane) was electrophoresed on a 7% SDS-polyacrylamide gel at 6 mA/gel for 14-16 h; the gel was then stained by silver (25). The location of protein standards is indicated on the ordinate (kilodaltons). The positions of the 180- and 170-kDa forms of topoisomerase II are indicated by p180 and p170, respectively. Panel C, the elution profile of p170. A duplicate gel to panel B was transferred to nitrocellulose and probed with anti-p170 antiserum (1:1000 dilution); the immunoreactive material was visualized with AuroProbe BL GAR.

FIG. 2. Mono S chromatography. Fractions from Fast Flow Q containing topoisomerase II activity were pooled and chromatographed on Mono S. Panel A shows the total protein pattern from each step of the purification as well as the peak fractions of topoisomerase II activity from Mono S. The locations of the 180- and 170-kDa forms of topoisomerase II are indicated by p180 and p170, respectively. The amount of protein loaded per lane was: A20 cells, 65 µg; 0.35 M extract, 24 µg; Fast Flow Q (F.F.Q) load, 18 µg; Mono S load, 2 µg; Mono S fractions, approximately 225 ng. In panel B, a duplicate gel to panel A was blotted onto nitrocellulose and probed with affinity-purified anti-p180 antibodies.

100-fold above the 0.35 M NaCl extract (Table I); this represents about a 500-fold purification of the enzyme from whole cells (assuming a 90% extraction of the enzyme). The relatively low fold purification may reflect loss of activity of the enzyme during purification, as the enzyme activity was found to decrease at 4 °C with a half-life of only several days, especially when the enzyme was in dilute solutions. The topoisomerase II activity of the purified preparation could be maintained for several months, however, when stored at −20 °C in 50% glycerol.

Topoisomerase II activity correlated very closely with the appearance of p170 and p180 (Fig. 3). The SDS-polyacrylamide gel shown in Fig. 24 was densitometrically scanned to give the relative amounts of p170 and p180 in each fraction (Fig. 3B, left panel), and each fraction was assayed for P4 unknotted activity (Fig. 3A). Topoisomerase II activity did not correlate with either p170 or p180 alone (Fig. 3B, left panel) but was very well correlated with the sum of the two proteins (Fig. 3B, right panel). An advantage of Mono S was that it provided sufficient separation of p170 from p180 to allow the two proteins to be assayed for topoisomerase II activity independently (compare fractions 10 and 11 with fractions 15 and 16). This type of analysis strongly suggests that both p170 and p180 possessed topoisomerase II activity.

Further evidence that both proteins have topoisomerase II activities is shown in Fig. 4, where specific polyclonal antibodies to p170 (described below) were used to inhibit P4 unknotted activity. Addition of anti-p170 antibodies to a sample containing primarily p180 (approximately 20% p170 as determined by densitometric scanning) resulted in only a slight inhibition of P4 unknotted activity (Fig. 4, A and B). When added to a sample containing only p170, however, the same amount of anti-p170 antibodies resulted in almost complete inhibition of P4 unknotted activity (Fig. 4, A and B). Inhibition of P4 unknotted activity with specific anti-p170 antibodies demonstrated that p170 possessed topoisomerase II activity. The inability to significantly inhibit activity in a sample containing primarily p180 clearly indicated that a second topoisomerase II activity was present. Although it appeared likely that the second topoisomerase II activity
photographed with Polaroid type 55 film; the amount of unknotted injected to this type of analysis, both proteins incorporated the
which takes advantage of the ability to trap an intermediate
The last two lanes in each set contain a knotted P4 DNA standard
and an unknotted P4 DNA standard (unknotted with excess purified
agarose gel and the DNA located by ethidium bromide. The gel was
of ~180, p180 was 0.1908.0, densitometric area of p170; 0, densitometric area
M, densitometric peak area. Areas are expressed as percent of maximum,
unknotting activity with the sum of p170 and p180. Densitometric
activity at dilutions of either 1:200 or 1:400 as described under
"Experimental Procedures." Samples were electrophoresed on a 0.7%
agarose gel and the DNA located by ethidium bromide. The gel was
densitometric scanning of the negative. The last two lanes in each set contain a knotted P4 DNA standard and an unknotted P4 DNA standard (unknotted with excess purified
topoiso merase II), respectively. B, left panel, correlation of P4 unknotting activity with the appearance of either p170 or p180. The amount of p170 or p180 in each fraction was quantitated by densitometric scanning of the gel shown in Fig. 2A and is expressed as densitometric peak area. Areas are expressed as percent of maximum, where the maximum area for p170 was 0.1506 and the maximum for p180 was 0.1908. , densitometric area of p170; O, densitometric area of p180; , P4 unknotting activity. B, right panel, correlation of P4 unknotting activity with the sum of p170 and p180. Densitometric areas of p170 and p180 were summed [L]; maximum area was 0.2674. , P4 unknotting activity.

resided in p180, this could not be determined immunologically, since anti-p180 had no effect on P4 unknotting activity of either p170 or p180 (data not shown).

To conclusively demonstrate that both p170 and p180 possessed topo ISO merase II activity, an experiment was done which takes advantage of the ability to trap an intermediate in the topo isomerase reaction in which the enzyme is covalently attached to DNA. As Rowe et al. (29, 30) previously demonstrated, denaturation of the complex formed between unlabeled topo isomerase II and 32P-labeled DNA results in covalent incorporation of 32P label into the topo isomerase II; by digesting the bulk of the DNA with Bal31 nuclease followed by SDS-PAGE and autoradiography, it can be determined which proteins in the sample mediate the DNA cleavage reaction. As shown in the left lane of Fig. 5, when a sample of purified topo isomerase II containing p170 and p180 was subjected to this type of analysis, both proteins incorporated the 32P label. In the absence of added topo isomerase II, no 32P bands were seen (data not shown). The addition of amsacrine

FIG. 3. Correlation of P4 DNA unknotting activity with p180 and p170. A, P4 unknotting activity of Mono S fractions 9–16. Fractions from Mono S (Fig. 2) were assayed for P4 unknotting activity at dilutions of either 1:200 or 1:400 as described under "Experimental Procedures." Samples were electrophoresed on a 0.7% agarose gel and the DNA located by ethidium bromide. The gel was photographed with Polaroid type 55 film; the amount of unknotted P4 DNA was quantitated by densitometric scanning of the negative. The last two lanes in each set contain a knotted P4 DNA standard and an unknotted P4 DNA standard (unknotted with excess purified topo iso merase II), respectively. B, left panel, correlation of P4 unknotting activity with the appearance of either p170 or p180. The amount of p170 or p180 in each fraction was quantitated by densitometric scanning of the gel shown in Fig. 2A and is expressed as densitometric peak area. Areas are expressed as percent of maximum, where the maximum area for p170 was 0.1506 and the maximum for p180 was 0.1908. , densitometric area of p170; O, densitometric area of p180; , P4 unknotting activity. B, right panel, correlation of P4 unknotting activity with the sum of p170 and p180. Densitometric areas of p170 and p180 were summed [L]; maximum area was 0.2674. , P4 unknotting activity.

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FIG. 4. Inhibition of P4 unknotting activity by anti-p170 antibody. A, fractions from Mono S containing predominantly p180 (approximately 20% p170 by densitometric scanning) or p170 were diluted into either preimmune IgG or dilutions of anti-p170 IgG in preimmune IgG as described under "Experimental Procedures." For each fraction, the highest enzyme dilution which would still give complete unknotting of the P4 DNA in 30 min at 37°C was chosen. After 3 h of incubation at 0°C with the antibody, the topo isomerase II activity of each enzyme was determined. Total assay volume was 10 μl; the volume of anti-p170 IgG in each assay is indicated above each lane. B, the amount of unknotted DNA in each lane of panel A was determined by densitometry. , fraction containing p170; ◆, fraction containing p180.

FIG. 5. Covalent transfer of 32P from DNA to enzyme. Purified topo isomerase II containing both p170 and p180 was incubated with [32P]dCTP-labeled DNA as described under "Experimental Procedures." The enzyme-DNA complex was denatured with SDS (1%) to trap the covalent intermediate, and the bulk of the DNA was digested by Bal31 nuclease. To determine which proteins had become covalently labeled with 32P, the sample was separated by SDS-PAGE and the dried gel exposed to Kodak XAR film for 18 h with an intensifying screen. The left lane shows covalent incorporation of 32P into p170 and p180; the right lane shows enhancement of this incorporation by 40 μg/ml amsacrine.
to the reaction caused an increase in the amount of \(^{32}P\) incorporated into the proteins (Fig. 5, right lane), as expected for topoisomerase II (30). Thus, it is clear that both p170 and p180 have topoisomerase II activity.

To determine the relationship between the two forms of topoisomerase II, two types of studies were undertaken. The first was a comparison of the proteolytic cleavage patterns of p170 and p180 by *Staphylococcus* V8 protease. Purified topoisomerase II fractions which contained approximately equal amounts of p170 and p180 were electrophoresed on a 7% SDS-polyacrylamide gel and located by Coomassie Blue staining; alternatively, radioiodinated topoisomerase II was included in the sample, and p170 and p180 were located by autoradiography of the wet gel. The individual bands were excised and
proteoloyzed in a second SDS-polyacrylamide gel according to the method of Cleveland et al. (31). As can be seen in Fig. 6, the proteolytic cleavage patterns of the two proteins were significantly different. Clearly, peptide fragments were produced by V8 protease digestion of p170 that were not generated by p180. Furthermore, intermediate fragments generated by low concentrations of V8 protease were also different between the two. Protein p170 was proteolyzed more readily by V8, but increasing amounts of V8 protease (up to 2.5 μg, data not shown) still produced many peptide fragments from each protein that were not formed from the other at any V8 amount. A low level of protein degradation was always observed in the absence of protease under the incubation conditions used (Fig. 6A, lanes 2 and 3); the cause of this proteolysis is not known. However, even this spontaneous proteolysis of the two proteins yielded distinctly different fragments.

The second type of comparison of p170 and p180 was done by analysis of their antigenic similarity. Specific polyclonal antibodies to either p170 or p180 were generated in rabbits and tested for their ability to cross-react with the different proteins. As described above (Fig. 4), antibodies to p170 were able to inhibit the catalytic activity of p170 but had very little, if any, effect upon p180 catalytic activity. As can be seen in Fig. 7A, lane B, antibodies raised against p170 were highly selective for that protein even when equal amounts of purified p170 and p180 were probed. Antisera against p180 was of lower titer but showed similar selectivity (data not shown); after affinity purification of the p180 antibody, it showed no cross-reactivity with p170 (Fig. 7A, lane C). The same specificity was observed when a 0.35 M NaCl extract of P388/A20 nuclei was probed (Fig. 7B), indicating that the antigenic differences observed in the purified preparations were not an artifact of the purification.

The results suggested that two forms of topoisomerase II were present in the P388/A20 cell line. Since this cell type was selected for its resistance to amsacrine, it was of interest to determine whether the P388/WT20 cells also possessed the p170 and p180 forms of topoisomerase II. Fig. 8 shows topoisomerase II purified from both cell types. Peak fractions from Mono S chromatography (fractions 10–15) were pooled and the samples run on SDS-PAGE and stained with silver. Comparison of the samples shows that each cell type contained both p170 and p180, though the ratio of the two bands was different. In each preparation, the relative amount of p170 was greater in the P388/WT20 cells. This ratio difference was also observed by immunoblotting of cell lysates that had been prepared by immediate lysis of the cells in boiling SDS (Fig. 9). This clearly demonstrated that p170 and p180 were present in the cells (and were not an artifact of extraction or purification) and indicated that the increased amount of p170 found in purified topoisomerase II preparations from P388/WT20 cells was not due to selective enrichment of p170 during purification. In addition, p170 and p180 antisera showed the same selectivity in the P388/WT20 as was demonstrated in P388/A20 (Fig. 9). Thus, while the relative amounts of two forms of topoisomerase II in the amsacrine-sensitive and -resistant P388 cells were different, it is clear that each cell type contained both forms.

**Discussion**

Topoisomerase II was purified from P388/A20 cells to near homogeneity by a rapid highly reproducible purification scheme. The procedure requires less than three days, reducing not only the amount of time devoted to enzyme purification, but also minimizing the loss of activity of this labile enzyme. From approximately 2 × 10^9 P388 cells, one can typically obtain several hundred micrograms of purified topoisomerase II. The availability of this quantity of purified enzyme has made it possible to generate antibodies which are highly specific for topoisomerase II.

The enzyme from P388/A20 cells was purified as two bands of 180 and 170 kDa on SDS-PAGE. Both forms of the enzyme unknotted P4 DNA in an ATP-dependent manner, an activity that can be accomplished only by a type II topoisomerase (22). Each form of the enzyme displayed amsacrine-stimulated covalent attachment to DNA. It appears highly unlikely that p170 is simply a proteolytic fragment of p180 for the following reasons: 1) fingerprinting analysis of the proteins by V8 protease revealed significantly different patterns; 2) specific polyclonal antibodies to the two proteins were highly selective for the protein used to generate the antibodies, both in inhibition of catalytic activity and in immunoblotting; 3) the ratio of the two proteins was consistent within a given cell type; 4) multiple protease inhibitors were used; 5) no conversion of p180 to p170 was ever observed, even after prolonged storage; 6) no other evidence of proteolysis was present, such as lower molecular weight forms; 7) the ratio of the two proteins was characteristic of a particular cell type; 8) both bands were present in cell lysates (in cells immediately lysed in boiling SDS).

P388 cells were chosen as the source of the enzyme for this study because of the availability of clones from both wild type P388 and an amsacrine-resistant mutant which may contain an altered topoisomerase II (20). The ability to purify the
enzyme from both amsacrine-resistant and -sensitive cells will allow comparison of the enzymatic properties of each and may lead to an understanding of the mechanism of amsacrine-induced topoisomerase II DNA cleavage and the development of resistance to this drug. Initial studies of the two forms of topoisomerase II have indicated that p170 is more sensitive than p180 to inhibition by amsacrine. Furthermore, preliminary evidence with specific antibodies to p170 and p180 demonstrates a correlation between the amount of p170 in a cell type and the cytotoxic potency of amsacrine. Thus, the relative levels of the two forms of topoisomerase II in a cell may be an important determinant of amsacrine sensitivity.

It is important to emphasize that the appearance of two forms of topoisomerase II is not limited to the amsacrine-resistant P388 cells. As shown in Figs. 8 and 9, the amsacrine-sensitive P388 cells also possess both enzymes. Specific antibodies to p180 recognize the same band in P388/WT20 as in P388/A20; antibodies to p170 behave similarly. Furthermore, preliminary immunoblotting experiments with specific antibodies to p170 or p180 have shown both forms of the enzyme to be present in lysates of other cell types as well, including human cells. Thus, it does not appear that one form of the enzyme is simply a mutant that developed during acquisition of amsacrine resistance.

Previous studies have also reported the purification of topoisomerase II, but no evidence was presented for different forms of the enzyme that was not due to proteolysis. Miller et al. (36) found only a 172-kDa topoisomerase from HeLa cells, and a 160-kDa topoisomerase II was isolated from Plasmodium berghei (37). Topoisomerase II from yeast was purified as a “polysisperse” band of 150 kDa, indicating to the authors that some proteolysis was occurring (38). In calf thymus, bands of 125 and 140 kDa were found, though these clearly represented proteolytic products of a 180-kDa form found by immunoblotting of cell lysates (39). Tryptic maps of the two proteins showed no significant differences (39). A more recent purification of the enzyme from calf thymus found protein bands of 175 and 150 kDa (40); while no comparison of the two bands was made, the authors did state that antibodies raised against the 175-kDa protein cross-reacted with the 150-kDa protein. Purification of the enzyme from Drosophila has resulted in the appearance of multiple bands on SDS-PAGE ranging from 172 to 132 kDa (41–43). As the authors point out, the most likely explanation for these multiple bands is proteolysis, since higher molecular weight species could be converted to the lower molecular weight species (41, 43), proteolytic cleavage patterns of the bands were very similar (41), and antibodies to the peptides showed cross-reactivity to the entire cluster of peptide bands (40, 41).

The reason that different forms of topoisomerase II have not been previously detected is not obvious. However, numerous differences exist between the present study and previous ones, including topoisomerase II extraction conditions, protease inhibitors, types of chromatography, and speed of the purification. Additionally, the type of cell used in the purification may be important. Preliminary results indicate that the amounts of p170 and p180 can be markedly different in different cell types; perhaps the cell types used in previous purifications contained so little of one form of topoisomerase II that it escaped detection.

It cannot be determined from the data whether the two forms of topoisomerase II are due to separate genes (or different splicing of one gene) or to post-translational modification of a single gene product. Several potential modifications of the enzyme have been demonstrated in vitro, including phosphorylation (44–46) and ADP-riboseylation (47), but these have not been found in vivo. A gene for topoisomerase II has been cloned from yeast (48) and Drosophila (49). In yeast it was shown by gene disruption to be a single copy essential gene (48). This finding does not exclude the possibility that more than one gene for topoisomerase II exists in mammalian cells, however. For example, yeast has a single copy metallothionein gene, while two copies of the gene are present in the mammalian genome (50). A topoisomerase II gene from mammalian cells has not as yet been isolated, so no data are available to exclude multiple genes. Additional studies will be required to determine the relationship of the two forms of topoisomerase II.

In light of the importance of topoisomerase II to cellular function, it is interesting to speculate what the roles of two forms of the enzyme may be. For example, the enzymes may have a differential subcellular distribution, such as in their association with the chromatin scaffold. Perhaps one of these enzymes associates with particular subsets of chromatin, such as regions which are actively transcribing or replicating. The quantity of topoisomerase II has been shown to be cell cycle-dependent (51); it may be that only one form of the enzyme displays this cell cycle dependence. The two enzymes could also differ in their enzymatic properties, such as cofactor requirements or substrate specificities. Previous studies of topoisomerase II have suggested that the enzyme is a homodimer (1–3); perhaps p170 and p180 may interact as a heterodimer under certain conditions. The ability to purify the enzymes in active form, as well as the availability of specific antibodies to the two proteins, will allow these questions to be addressed.

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