Biochemical and Pharmacological Properties of p170 and p180 Forms of Topoisomerase II†

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ABSTRACT: The p170 and p180 forms of topoisomerase II have been compared. The concentration dependence of ATP for catalytic activity of the two forms of the enzyme was identical, and each was equally sensitive to novobiocin. Orthovanadate was found to be a potent inhibitor of catalytic activity of both p170 and p180, with an IC_{50} value of about 2 μM for each. Under standard reaction conditions, relaxation of supercoiled pBR322 by p180 was highly processive, while p170 performed the same reaction in a distributive manner. The optimal concentration of KCl for catalytic activity of p180 was 20–30 mM higher than that for p170. Comparison of their thermal stability showed that p180 was inactivated at twice the rate of p170. Teniposide and merbarone selectively inhibited catalytic activity of p170, requiring concentrations 3-fold and 8-fold lower, respectively, than those required for equivalent inhibition of p180. Similar selectivity for p170 was seen for teniposide-stimulated DNA cleavage or its inhibition by merbarone. Analysis of sites of DNA cleavage indicated a subset of sites that were either preferred or unique for each of the enzymes. A synthetic oligonucleotide representative of p170 sites selectively inhibited the p170 enzyme. Immunoblotting of p170 and p180 from U937 cells at different stages of proliferation showed that p170 levels declined as the cells reached the plateau phase of growth, while p180 levels were low during rapid proliferation and increased as the growth rate slowed. The data indicate that the p170 and p180 forms of topoisomerase II can be distinguished biochemically, pharmacologically, and by differential cellular regulation.

Type II topoisomerases change DNA topology by passing one strand of DNA through a reversible break in a second DNA strand [for reviews, see Wang (1985), Maxwell and Gellert (1986), and Vosberg (1985)]. Conditional mutation of the top2 gene in yeast has shown that this enzymatic activity is required for segregation of daughter chromosomes following replication (Uemura & Yanagida, 1986; DiNardo et al., 1984; Holm et al., 1985). Indirect evidence suggests a role for this class of enzyme in initiation of DNA replication (Nelson et al., 1986; Zhang et al., 1986), transcription (Kmiec et al., 1986; Schroder et al., 1987; Hirose & Suzuki, 1988), and DNA recombination (Pommier et al., 1988; Bae et al., 1988).

Previous data from our laboratory demonstrated that topoisomerase II activity from P388 leukemia cells resided in polypeptides of 170 and 180 kDa (Drake et al., 1987). Each enzyme unknotted P4 DNA in an ATP-dependent manner and displayed amsacrine-stimulated covalent binding to DNA. The two forms of the enzyme were antigenically distinct, as judged by immunoblotting and by inhibition of catalytic activity with immune sera. Proteolytic digestion of the enzymes produced some peptides that appeared to be common to both forms but also generated a number of peptides that were unique. Each form of the enzyme was present in whole cell lysates; in addition, the relative amount of the two forms of the enzyme differed in amsacrine-resistant P388 cells compared to its parental line (Drake et al., 1987; Per et al., 1987). The data strongly suggested that the p170 and p180 forms of topoisomerase II are distinct enzymes.

To begin to understand how the two forms of topoisomerase II differ, and perhaps gain insight into their cellular role(s), the enzymatic and pharmacological properties of p170 and p180 have been compared. The data demonstrate that the enzymes can be distinguished both pharmacologically and enzymatically and that the two forms are differentially regulated.

Experimental Procedures

Materials. Teniposide and merbarone were generously provided by the Drug Synthesis and Design and Natural Products Branches of the National Cancer Institute, Bethesda, MD. All other materials were as previously described (Drake et al., 1987).

Purification of p170 and p180 Forms of Topoisomerase II. P388 (Wt20 or A20) cells were grown interperitoneally in mice as previously described (Per et al., 1987). U937 (human monoblast like) cells were grown in 3-L spinner flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. Topoisomerase II was purified by a modification of the method of Drake et al. (1987). Pooled fractions from hydroxylapatite were applied without dilution to a 1.0 × 1.5 cm column of phenyl-Sepharose and eluted with a descending gradient of 0.75 M potassium phosphate to 0 M potassium phosphate (20 mM Tris-HCl), each containing 10% glycerol and 10 mM 2-mercaptoethanol. Fractions from phenyl-Sepharose containing topoisomerase II activity were applied to a Mono Q column as described (Drake et al., 1987). Fractions from Mono Q were stored at −20 °C in 50% glycerol. The experiments described were done with p170 and p180 purified

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from P388 cells (Wt20) or the amsacrine-resistant clone (A20). Studies of inhibition of catalytic activity of the enzymes by teniposide and merbarone were also conducted with the enzymes purified from U937 cells.

Topoisomerase II Catalytic Assays. Topoisomerase II catalytic activity was assayed by the ATP-dependent unknotting of phage P4 DNA (Liu et al., 1981). To quantify the amount of unknotted DNA produced, photographic negatives of the ethidium bromide stained agarose gels were densitometrically scanned as previously described (Hofmann et al., 1987). For the quantitative P4 unknotting assay, standard errors typically represent 5–10% of the mean. Alternatively, topoisomerase II catalytic activity was assayed by the ATP-dependent relaxation of plasmid pBR322. Assays were performed in a total volume of 10 μL containing 165 ng of supercoiled pBR322, 50 mM Tris-HCl, pH 7.5, 100 mM KCl (or the concentration indicated), 10 mM $MgCl_2$, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 30 μg/mL bovine serum albumin, 1 mM ATP (or the indicated concentration), and approximately 1 unit of enzyme (defined as the amount of enzyme required to relax 50% of the DNA in 30 min under these reaction conditions). After 30 min at 37 °C, the reaction was stopped by the addition of SDS[1] (to a final concentration of 1%)/Ficoll/bromophenol blue. Under these conditions, the rate of the reaction was linear for at least 45 min. The samples were electrophoresed at 2.5 V/cm for 12 h in 1% agarose in Tris/borate/EDTA buffer. Activity was quantified from disappearance of supercoiled substrate by densitometric scanning of photographic negatives (Osheroff et al., 1983). Data presented for catalytic activity (Figures 2–4, 5A, 6, and 8) are representative examples of at least four separate experiments.

DNA Cleavage Assays. Topoisomerase II mediated DNA cleavage was measured by the conversion of supercoiled plasmid pBR322 to its linear form (Liu et al., 1983). The reaction conditions were the same as for relaxation of supercoiled pBR322, except that 20–40 units of enzyme were used along with the indicated concentrations of drug. Prior to electrophoresis, samples were treated with 1 mg/mL protease K in 1% SDS for 30 min at 37 °C. Cleavage was measured by appearance of linear pBR322 by densitometric scanning of photographic negatives. For determination of teniposide-stimulated DNA cleavage sites, the cleavage of [^{32}P]phosphate-labeled linear pBR322 was assayed according to the method of Liu et al. (1983).

SDS–PAGE and Immunoblotting. U937 cells were seeded at 0.8 × 10^5/mL and grown in flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. Cells were counted by hemocytometer, and viability was determined by trypan blue exclusion. At the indicated times the cells were centrifuged at 1000*g* and the cell pellet washed in ice-cold phosphate-buffered saline containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 50 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor). The cells were immediately lysed by addition of hot (68 °C) 2% SDS in phosphate-buffered saline (containing the same protease inhibitors), and the lysed cells were heated at 68 °C for 5 min. SDS–PAGE was performed on 7% polyacrylamide gels by the method of Laemmli (1970); each lane contained equal amounts of protein (BCA protein assay, Pierce Chemical Co.). Gels were transferred to Immobilon (Millipore) at 400 mA for 45 min in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11 (Matsudaira, 1987), in a Hoeffer transfer unit. Antibodies to p170 and p180 were raised in rabbits by intradermal injection of keyhole limpet hemocyanin-conjugated synthetic peptides whose sequences were based upon the predicted amino acid sequences of the two classes of topoisomerase II cDNA clones (Tan et al., 1988) which code for the p170 and p180 enzymes.[2] For affinity purification of the antibodies, the synthetic peptides were immobilized to Affiprep 10 (Bio-Rad) according to manufacturer's instructions. Immunoblotting was as previously described (Drake et al., 1987), except that 0.5% Tween 20 was used as the blocking agent and visualization was done with goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega Biotech).

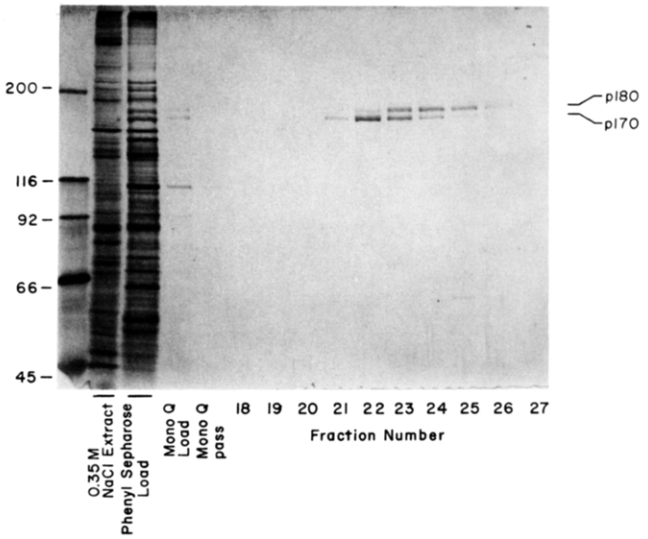

FIGURE 1: Purification of topoisomerase II. Nuclear extract (0.35 M NaCl) was purified sequentially over Ultrogel hydroxylapatite, phenyl-Sepharose, and Mono Q. Fractions from Mono Q were electrophoresed on SDS–PAGE and stained with silver. The locations of p170 and p180 are indicated. The locations of protein standards are indicated on the ordinate (kilodaltons).

Results

To compare the p170 and p180 forms of topoisomerase II, the purification scheme was optimized for separation of the enzymes. The best separation of p170 and p180 was obtained with Mono Q. Phenyl-Sepharose, while not separating the enzymes, provided a greater purification of the enzymes without the need for large dilution of the pooled hydroxylapatite fractions. Therefore, the purification procedure (Drake et al., 1987) was modified so that phenyl-Sepharose was inserted as the second chromatographic step, followed by Mono Q. Figure 1 shows an example of the enzyme purity and separation following Mono Q. These results were obtained consistently with numerous enzyme preparations and different columns. Fractions from the leading edge of the topoisomerase II peak contained the p170 form of the enzyme, followed by a mixture of two forms, and then fractions containing the p180 form. For the enzyme studies to be described, only fractions containing greater than 90% of p170 or p180 were used.

Previously published data showed that p170 and p180 unknotted P4 DNA in a rection that required ATP (Drake et al., 1987). Figure 2 demonstrates that the concentration dependence of ATP for catalytic activity of the two forms of

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[2] T. D. Y. Chung, F. H. Drake, K. B. Tan, S. R. Per, S. T. Crooke, and C. K. Mirabelli. Characterization and Immunological Identification of cDNA Clones Encoding Two Human Topoisomerase II Isozymes. Submitted for publication.

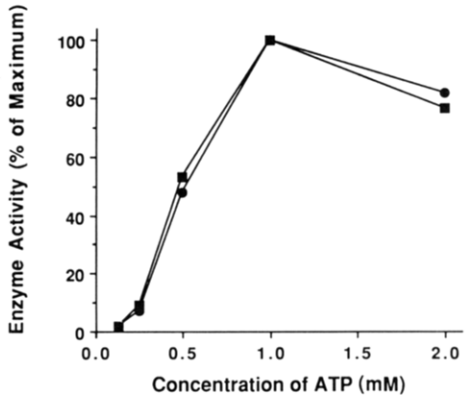

FIGURE 2: Concentration dependence of ATP for catalytic activity of p170 and p180. Catalytic activity of p170 (■) and p180 (●) was assayed by relaxation of supercoiled plasmid pBR322 at the indicated concentrations of ATP. Enzyme activity is expressed as the percentage of the maximum activity obtained.

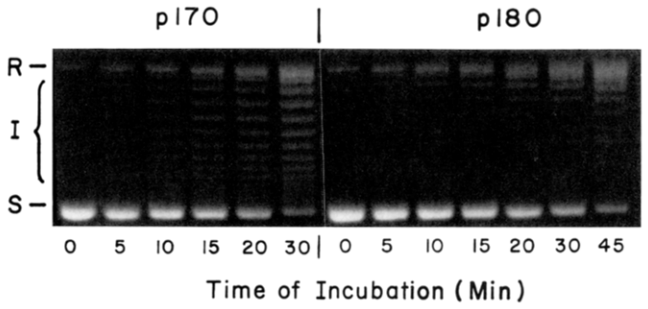

FIGURE 3: Distributive vs processive relaxation of supercoiled pBR322. Time course of relaxation of supercoiled plasmid pBR322. Supercoiled pBR322 (S) was converted to relaxed plasmid (R) by either p170 or p180. The reaction was stopped at the indicated times. (I) Topoisomers of intermediate superhelicity.

the enzyme was virtually identical. Both were inactive at low ATP, had optimal activity about 1 mM ATP, and had decreased activity at higher concentrations of ATP. The enzymes were equally sensitive to novobiocin, an ATP-competitive inhibitor of topoisomerase II (Osheroff et al., 1983), with IC_{50} values of about 200 μM. Orthovanadate is another compound that inhibits some ATPases, most notably ion-translocating enzymes such as the Na/K ATPase (Nechay et al., 1986). Orthovanadate was a potent, nonselective inhibitor of p170 and p180, with IC_{50} values of about 2 μM for each enzyme.

Although both forms of the enzyme catalyzed DNA strand passage in an ATP-dependent manner, the progress of the reaction was quite different for p170 and p180 under standard reaction conditions. This was demonstrated by following the time course for relaxation of supercoiled plasmid pBR322 (Figure 3), which has approximately 30 superhelical turns (Osheroff et al., 1983). Topoisomerase II changes the linking number in steps of 2 (Wang, 1985; Maxwell & Gellert, 1986; Vosberg, 1985), so that approximately 15 strand passage events are required to relax the plasmid fully (Osheroff et al., 1983). Under completely distributive conditions, the enzyme relaxes only one supercoil before dissociation and topoisomers of intermediate superhelicity accumulate. In contrast, under processive conditions the enzyme completely relaxes all the supercoils before dissociation, so that intermediates do not accumulate. As shown in Figure 3, p170 relaxed pBR322 in a highly distributive manner under standard reaction conditions (100 mM KCl). Very little fully relaxed plasmid was present until nearly all of the supercoiled substrate had been depleted, and a number of intermediates were observed. Under the same reaction conditions, p180 behaved in a processive manner (Figure 3). Fully relaxed plasmid appeared at a time when very little substrate had been depleted, and few intermediates were observed.

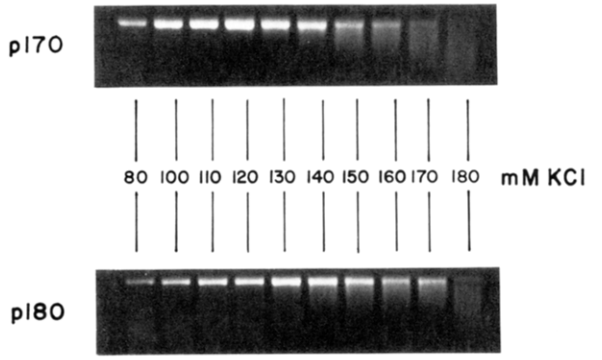

FIGURE 4: Effect of KCl on catalytic activity of p170 and p180. P4 unknotting activity of p170 (upper panel) and p180 (lower panel) was assayed at the indicated concentrations of KCl. The enzymes convert knotted P4 DNA (smear) to its unknotted form (band).

A key factor in processivity of topoisomerase II is the concentration of salt in the reaction (Osheroff et al., 1983). At lower concentrations of salt the enzyme is more processive, while higher salt concentrations make the enzyme behave more distributively (Osheroff et al., 1983). In the experiment in Figure 3, each enzyme was tested at the same salt concentration. However, both p170 and p180 can be made to behave more processively or distributively by adjusting the salt concentration (data not shown). As shown in Figure 4, however, the optimum concentration of KCl was higher for the p180 form of the enzyme than for p170. The optimal concentration of KCl for unknotting of P4 DNA by p170 was about 110 mM, as judged by maximal product formation (unknotted band) and essentially no substrate remaining (knotted smear). As the KCl concentration was increased, there was the appearance of increasing amounts of substrate, and by 160 mM KCl the enzyme was virtually inactive. In contrast, maximal product formation for p180 occurred at 130–140 mM KCl, and significant activity was present up to 170 mM KCl (Figure 4).

The p170 and p180 forms of topoisomerase II also appeared to differ in stability. Under identical storage conditions, p180 activity consistently decreased at a faster rate. Studies of thermal stability of the enzymes confirmed this difference. Preincubation of the enzymes at 25 °C in the absence of ATP or DNA inactivated the p170 form of the enzyme with a half-time of approximately 10 min (data not shown). Under identical conditions, catalytic activity of p180 decreased with a half-time of only 5 min.

A number of antineoplastic drugs have been reported to be inhibitors of topoisomerase II [see Glisson and Ross (1987)]. Several of these drugs were tested against p170 and p180 to determine whether the two forms were pharmacologically distinguishable. As shown in Figure 5A, for equivalent inhibition of catalytic activity, the p180 form of the enzyme required an approximately 3-fold higher concentration of teniposide than the p170 form. Similar results were seen with the enzymes purified from human (U937) cells grown in culture (data not shown). The mechanism of inhibition of teniposide has been reported to be stabilization of a covalent enzyme–DNA intermediate (Ross et al., 1984). This intermediate can be trapped by denaturation of the enzyme–DNA complex, resulting in a topoisomerase-associated DNA strand break (Ross et al., 1984). As shown in Figure 5B, teniposide enhanced topoisomerase II breakage of DNA. However, as was the case for inhibition of catalytic activity, higher concentrations of teniposide were required to induce p180-me-

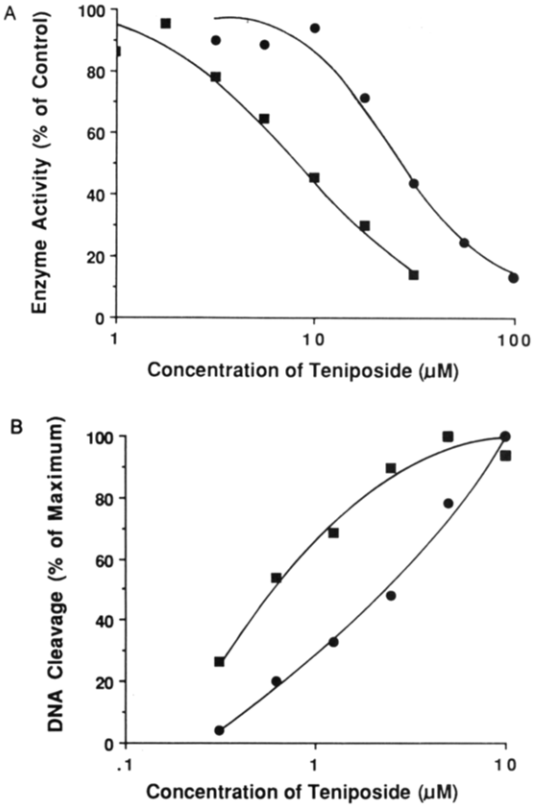

FIGURE 5: Effect of teniposide on catalytic activity and DNA cleavage activity of p170 and p180. (Panel A) P4 unknotting activity of p170 (■) and p180 (●) was assayed in the presence of the indicated concentrations of teniposide. Activity is expressed as percentage of control, i.e., in the absence of teniposide. (Panel B) DNA cleavage activity of p170 (■) and p180 (●) was measured by the conversion of supercoiled pBR322 to its linear form in the presence of the indicated concentrations of teniposide. DNA cleavage is expressed as a percentage of the maximum obtained for each enzyme. The enzymes were of equal specific activity for P4 unknotting and were adjusted to give an equal number of units in each assay.

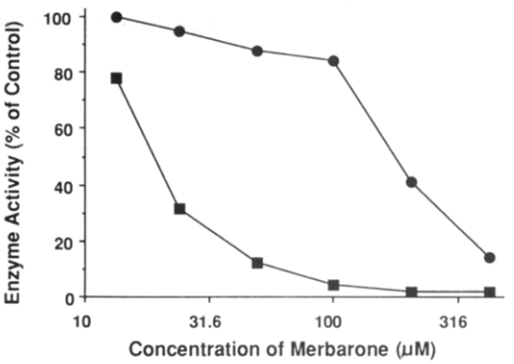

FIGURE 6: Effect of merbarone on catalytic activity of p170 and p180. The P4 unknotting activity of p170 (■) and p180 (●) was assayed in the presence of the indicated concentrations of merbarone. Activity is expressed as the percentage of the unknotting obtained for each enzyme in the absence of merbarone.

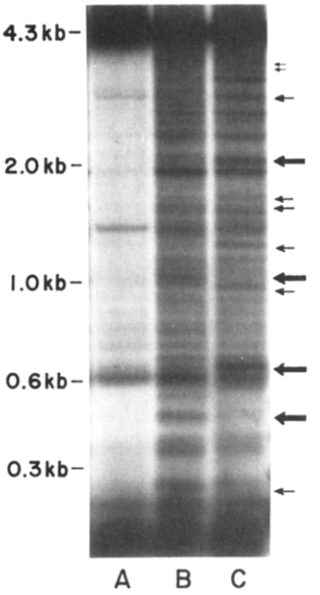

FIGURE 7: Teniposide-stimulated DNA cleavage sites of p170 and p180. Plasmid pBR322 was linearized and end-labeled with [^{32}P]-phosphate. The DNA was cleaved by either p170 (lane B) or p180 (lane C) in the presence of teniposide, and the samples were electrophoresed in 1.2% agarose and autoradiographed. Concentrations of teniposide were adjusted to give approximately eqivalent DNA cleavage activities for the two enzymes. Lane A shows the DNA substrate in the absence of enzyme or drug. Arrows indicate cleavage site differences; the size of the arrow indicates the approximate magnitude of the difference between the two enzymes.

diated DNA strand breakage than those required to induce p170-mediated breaks. Unlike the relative potency differences seen for inhibition of catalytic activity, which were very reproducible, the relative concentrations required for stimulation of DNA cleavage by the two enzymes were variable (from 3-fold to about 25-fold more sensitive for p170). The variability was due in part to different methods used to assay for DNA strand breaks but also appeared to be due to variation between enzyme preparations. However, under conditions where the amounts and activities of p170 and p180 were most closely matched, the differences appeared to be about 4-fold. While the relative potency differences for stimulation of cleavage activity varied, the p170 form of the enzyme was always more sensitive to the effects of teniposide.

Merbarone appears to represent a class of inhibitor that is different from the "cleavable complex" inhibitors of topoisomerase II, such as teniposide (Drake et al., 1989). As shown in Figure 6, much higher concentrations of merbarone were required to inhibit catalytic activity of p180 than p170. The IC_{50} of merbarone for p170 was approximately 20 μM, while the IC_{50} for p180 was approximately 175 μM; similar results were obtained with the enzymes from U937 cells (data not shown). Merbarone does not enhance topoisomerase II mediated DNA strand breakage and prevents teniposide-stimulated DNA cleavage by p170 (Drake et al., 1989). However, consistent with the potency differences seen for inhibition of catalytic activity, higher concentrations of merbarone were required to prevent teniposide-stimulated DNA cleavage activity by p180 than for p170 (data not shown).

In addition to potency of teniposide for p170 and p180, the location of drug-induced cleavage sites was also examined. Plasmid pBR322 was linearized by restriction enzyme and end-labeled with ^{32}P (Liu et al., 1983). After incubation of enzyme, labeled DNA, and teniposide, the reaction was stopped with SDS and the enzyme was proteolyzed with protease K (Liu et al., 1983). The labeled DNA fragments were then separated by electrophoresis and visualized by autoradiography. As demonstrated in Figure 7, at approximately equal amounts of drug-stimulated DNA cleavage by the two enzymes, a number of cleavage sites were observed for each enzyme. Some of the cleavage sites appeared to be common to both enzymes, but a subset of the sites were either preferred or unique for each of the two forms.

Although the exact sequence of the cleavage sites could not be determined by this analysis, mapping of the sites to the known sequence of pBR322 and computer analysis of the unique sites suggested that the p170 form of the enzyme

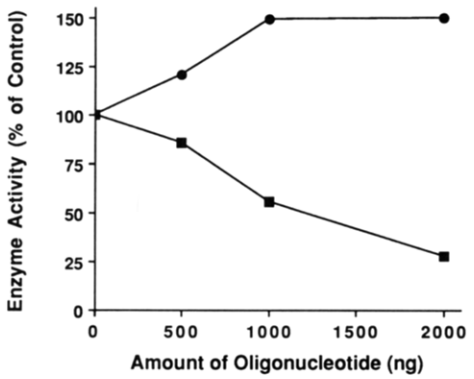

FIGURE 8: Effect of A·T-rich oligonucleotide on catalytic activity of p170 and p180. A 27-bp oligonucleotide was synthesized with the sequence 5′-TTTAAAATTTAAAATTTAAAATTTAAA-3′ and annealed with its complementary strand. P4 unknotting activity of p170 (■) and p180 (●) was measured in the presence of the indicated amount of the oligonucleotide. Activity is expressed as the percentage of unknotting obtained in the absence of added oligonucleotide.

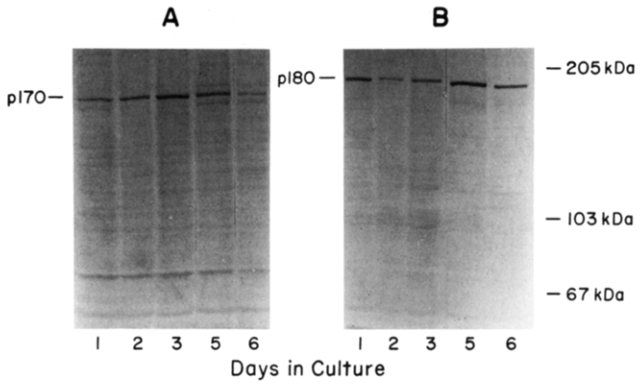

FIGURE 9: Proliferation dependence of p170 and p180. U937 cells were seeded (day 0) at 0.8×10^5/mL in RPMI medium containing 10% fetal calf serum. At the indicated times in culture, aliquots were removed and lysed in 2% SDS. Equal protein samples were electrophoresed by SDS–PAGE and electroblotted onto Immobilon. Duplicate halves of the blot were probed with affinity-purified antibodies to p170 (A) or p180 (B). The density of cells and viability for each day were as follows: day 1, 1.0×10^5/mL, 80% viable; day 2, 1.5×10^5/mL, 80% viable; day 3, 5.1×10^5/mL, 92% viable; day 4, 9.8×10^5/mL, 94% viable; day 5, 1.1×10^6/mL, 82% viable; and day 6, 9.0×10^5/mL, 67% viable.

preferred A·T-rich areas, while p180 preferred a G·C-rich consensus sequence. To test this, an A·T-rich oligonucleotide was synthesized and tested for its ability to inhibit catalytic activity of either p170 or p180. As shown in Figure 8, concentrations of the oligonucleotide that inhibited catalytic activity of p170 had no inhibitory effect upon p180 and actually slightly increased its activity. A G·C-rich oligonucleotide of the same length was equally effective at inhibiting p170 and p180 catalytic activity (data not shown).

Previously published data demonstrated that the amounts of topoisomerase II can vary during the cell cycle and according to proliferation rate (Heck & Earnshaw, 1986; Sullivan et al., 1987; Chow & Ross, 1987; Heck et al., 1988; Fairman & Brutlag, 1988; Hsiang et al., 1988). To compare the expression of p170 and p180 during changes in proliferation rate, U937 cells were grown for various times in culture and the levels of p170 and p180 were analyzed by immunoblotting (Figure 9); equal amounts of protein were loaded in each lane. As shown in Figure 9, panel A, the level of expression of p170 was highest on days 2 and 3, when the cells were in logarithmic growth, and dropped markedly as the cells reached plateau phase of growth (days 5 and 6). In contrast, as shown in panel B, the amount of p180 was low during logarithmic growth and increased significantly as the cells reached plateau phase. For P388 cells, p170 levels did not appear to change as the cells slowed in growth rate, but p180 levels increased as the growth rate declined (data not shown).

Discussion

Previously published data demonstrated that p170 and p180 were type II topoisomerases that differed in antigenic properties, proteolytic fragmentation patterns, and relative amounts in cells made resistant to topoisomerase II drugs (Drake et al., 1987). The present study extends these observations by demonstrating that p170 and p180 can be distinguished both by their biochemical and pharmacological properties and by differential regulation as a function of cellular proliferation rate.

Comparison of p170 and p180 revealed several significant differences but also some similarities. The two forms of the enzyme showed a nearly identical ATP concentration dependence and were equally inhibited by novobiocin, a competitive inhibitor of ATP (Osheroff et al., 1983). Orthovanadate, previously shown to be an inhibitor of some types of ATPases (Nechay et al., 1986), is demonstrated for the first time to be a potent inhibitor of topoisomerase II. Like novobiocin, vanadate inhibited the two forms equally. Vanadate potently inhibits enzymes that have phosphoenzyme intermediates (Nechay et al., 1986) and may inhibit p170 and p180 at their ATP sites. However, it is also possible that it interferes with the tryosine-mediated cleavage and reunion reaction with DNA in a manner analagous to its inhibition of phosphotyrosine phosphatases (Swarup et al., 1982a,b). Further studies will be required to determine the mechanism of inhibition by vanadate.

Each enzyme unknotted P4 DNA and relaxed supercoiled pBR322, but the reaction was more processive with p180 than p170. This suggests that p180 has either a more rapid catalytic rate or a slower dissociation rate from the DNA (Osheroff et al., 1983). It seems likely it is due to a slower dissociation rate, since high salt concentrations dissociate the enzyme from DNA (Osheroff, 1986), but p180 was active at a higher concentration of KCl than p170.

The sensitivity of the two forms of topoisomerase II to drugs also differed, both for inhibition of catalytic activity and for enzyme-mediated DNA cleavage. Teniposide showed a consistent 3-fold selectivity for p170 compared to p180 for inhibition of catalytic activity. Merbarone was even more selective for p170, with an 8- to 10-fold difference in potency for inhibition of catalytic activity. These differences in enzyme sensitivity were also observed with p170 and p180 purified from a human cell line (U937), demonstrating that this is not a property which is unique to the enzymes from P388 cells. Comparison of DNA cleavage activity of p170 and p180 was technically more difficult, because cleavage is stoichiometric rather than catalytic, requiring one enzyme for each cleavage event (Wang, 1985). While catalytic assays only measure fully catalytically competent enzyme, cleavage assays may also measure enzyme that is not catalytically active but is still able to cleave. Thus, the amount of each enzyme, as well as its specific cleavage activity, determines the amount of drug-stimulated DNA cleavage. The potency difference for teniposide-stimulated cleavage varied depending upon the assay used and the enzyme preparation, but p170 was consistently more sensitive to the drug. In experiments where the two enzymes were most closely matched in catalytic activity, amount of enzyme, and maximal DNA cleavage activity, the potency difference for teniposide was about 4-fold, which is similar to the difference observed in catalytic activity.

Merbarone was able to decrease the teniposide-stimulated DNA cleavage activity of the enzymes; however, consistent with inhibition of catalytic activity, higher concentrations of merbarone were required to inhibit cleavage by p180.

The difference in sensitivity of p170 and p180 to teniposide may explain the changes in enzyme ratio observed in P388/A20, an amsacrine-resistant subline of P388 that is also resistant to teniposide (Per et al., 1987). As previously reported (Drake et al., 1987; Per et al., 1987), the amount of p170 was decreased in P388/A20 compared to its parental line, while p180 levels remained the same in both. Selective loss of the most drug-sensitive enzyme would suffice to confer a level of resistance to the drug. This also suggests that increased drug resistance could be obtained by reduction of both p170 and p180 by the cell.

In addition to differences in drug sensitivity, the two enzymes also showed different patterns of teniposide-stimulated cleavage sites. Some of the sites appeared to be common to both forms of the enzyme, while other cleavage sites were unique for either p170 or p180. In the absence of drug, the amount of cleavage by the two enzymes was below the limits of detection for the assay. Computer analysis of the drug-stimulated cleavage data suggested that p170 preferred A·T-rich sequences, while p180 preferred a G·C-rich sequence virtually identical with that recently reported by Fosse et al. (1988). On the basis of the data, synthetic oligonucleotides of these sequences were used to compete for enzymatic activity of the two enzymes. The p170 enzyme was inhibited by both oligonucleotides, while p180 was inhibited only by the G·C-rich oligonucleotide. Thus, even in the absence of drug, p170 and p180 were differentially sensitive to oligonucleotides which differed only in sequence. The data suggest that p170 and p180 bind to different regions of the DNA. Furthermore, since p170 did not discriminate between the two types of oligonucleotides, it is possible that this enzyme is less selective in its recognition of DNA sequences. This could contribute to the more distributive behavior of this enzyme. If the p170 enzyme binds to DNA sites with lower affinity than p180, its rate of dissociation would be faster (assuming similar association rates). Thus, during the course of multiple rounds of relaxation of pBR322, the p170 enzyme would tend to dissociate more often and produce topoisomers of intermediate superhelicity.

Another important difference between p170 and p180 was the regulation of their amounts during cellular proliferation. The amount of p170 dropped sharply as the cells reached plateau phase, while the amount of p180 actually increased at plateau. This reciprocal regulation was even somewhat apparent on day 1 (i.e., 24 h after seeding) when the cells were not yet in logarithmic growth. The data for p170 are consistent with previously reported data for topoisomerase II, which show that the amounts of the enzyme either remain constant or decrease when cells reach the stationary phase of growth (Heck & Earnshaw, 1986; Sullivan et al., 1987; Fairman & Brutlag, 1988; Hsiang et al., 1988). For example, Sullivan et al. (1987) demonstrated that nuclear extracts of quiescent CHO cells contained much less topoisomerase II than extracts from logarithmically growing CHO cells. In L1210 cells, however, topoisomerase II amounts were similar in logarithmically growing cells and cells in plateau phase (Sullivan et al., 1987). The p170 enzyme from U937 cells behaves similarly to the topoisomerase II shown for CHO cells, while the p170 enzyme in P388 cells appears to be regulated like the enzyme from L1210 cells (Sullivan et al., 1987). This suggests that immunoblotting of topoisomerase II in other laboratories was done with antibodies to the p170 form of the enzyme. The p180 form of the enzyme in U937 cells is the first reported example of topoisomerase II levels increasing as the cell growth rate declines. In addition, recent data from our laboratory have shown that the relative increase in p180 levels compared to p170 can be seen more dramatically in U937 cells that have been induced to terminally differentiate by treatment with the phorbol ester TPA.[3]

The thermal stability of the two forms of the enzyme also appeared to differ, with p180 inactivated at about twice the rate of p170 at 25 °C. This suggests that the enzymes differ in structure. In addition, the data suggest that the enzymes are significantly stabilized by substrates, since the catalytic reaction is linear for at least 45 min under these conditions.[4] The difference in stability of the enzymes, as well as their proliferation dependence, could also help to explain why two forms of the enzyme were not reported by earlier investigators. Selective loss of p180 activity, especially if the cells were harvested at a growth stage where the amount of p180 was substantially lower than p170, would give the appearance of only a single form of the enzyme.

As pointed out by Lynn et al. (1986), eukaryotic topoisomerase II appears to be divided into domains: an amino-terminal end homologous to the ATP binding subunit of gyrase; a central portion containing the breakage–reunion tyrosine; and a carboxy-terminal DNA binding domain homologous with histone H1. On the basis of this functional assignment, the data presented here for p170 and p180 suggest that the difference(s) between the two forms may be greatest at the carboxy-terminal end of the proteins. The two forms of the enzyme may share a common or highly conserved ATP binding domain, since ATP and novobiocin do not show preference for either enzyme. The central domain, which is involved with DNA cleavage, may differ for p170 and p180, as judged by sensitivity to drugs that affect this reaction. The C-terminal domain seems to be the most different, as suggested by the different DNA cleavage sites, differential inhibition by A·T-rich oligonucleotides, and different DNA dissociation rates as suggested by the more processive behavior of p180.

The differences in the DNA sequence preference of p170 and p180, as well as their differential regulation during proliferation, lead to the interesting speculation that the enzymes may perform different functions in the cell. For example, topoisomerase II has been proposed to be a structural component of the nuclear scaffold (Earnshaw et al., 1985; Berrios et al., 1985; Earnshaw & Heck, 1985; Gasser et al., 1986), where it binds to A·T-rich regions of DNA (Mirkovitch et al., 1984; Gasser & Laemmli, 1986)). Since A·T-rich oligomers were unable to compete for catalytic activity of p180, it is possible that p170 preferentially associates with nuclear scaffold. Adenine–thymine tracts have also been suggested to be components of replication origins (Deb et al., 1986; Umek & Kowalski, 1988). The high levels of p170 present while cells are actively dividing may be due to its involvement in replication initiation. The finding that p180 levels increased as the cells slowed their growth suggests a different role for this enzyme. For example, p180 may not be required for chromosomal segregation. In addition, G·C-rich areas of DNA have been found to be present in promoter regions of genes for a number of "housekeeping" proteins, such as $G_s\alpha$ (Kozasa

[3] M. R. Mattern, F. H. Drake, K. B. Tan, S.-M. Mong, J. O. Bartus, C. K. Mirabelli, and S. T. Crooke. Roles of Topoisomerases I and II in the Differentiation of Human U937 Monoblast Cells. Submitted for publication.

[4] F. H. Drake, unpublished observations.

et al., 1988). Perhaps p180 has a role in transcription. Nucleolar DNA is also G·C-rich (Willems et al., 1968). It is possible that p180 is involved with transcription of ribosomal RNA.

The data presented demonstrate that the p170 and p180 forms of topoisomerase II possess unique biochemical properties, have differential pharmacological sensitivity to several antineoplastic drugs, and are differentially regulated during cellular proliferation. These differences may provide a means of designing antineoplastic agents that are more selective for p170 or p180 and that may possess novel spectra of pharmacological activity.

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