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Review

Separation and functional analysis of eukaryotic DNA topoisomerases by chromatography and electrophoresis

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Abstract

DNA topoisomerases are enzymes that control DNA topology by cleaving and rejoining DNA strands and passing other DNA strands through the transient gaps. Consequently, these enzymes play a crucial role in the regulation of the physiological function of the genome. Beyond their normal functions, topoisomerases are important cellular targets in the treatment of human cancers. In this review we summarize current protocols for extracting and purifying DNA topoisomerases, and for separating subtypes and isoforms of these enzymes. Furthermore, we discuss methods for measuring the catalytic activity of topoisomerases and for monitoring the molecular effects of topoisomerase-directed antitumor drugs in cell-free assays.

Keywords: Reviews; Enzymes; DNA topoisomerases; Topoisomerases

Contents

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1. Introduction

The structure of the double helix together with the nuclear organization of the DNA into closed loop domains implies that virtually every physiological function of the genome is modulated by topological relationships within the DNA [1,2]. In the cell, DNA topology is regulated and controlled by ubiquitous enzymes known as topoisomerases, which are required for important DNA processes such as replication, transcription [3,4], recombination [5], repair [6] chromosome (de)condensation and sister chromatid segregation [7–9]. Topoisomerases are characterized by their ability to break and reseal the polyphosphate backbone of the DNA and to pass other strands of DNA through the transient gaps [7,10,11]. By this action they can unwind, unknot, untangle, and, thus, resolve complex DNA structures [1,12]. There are two types of DNA topoisomerase, with different physico-chemical and catalytic properties. Type I topoisomerases are monomeric proteins of 100 g/mole which function without the input of metabolic energy. They can alter the pitch of DNA double helices by cutting one DNA strand and allowing the passage of a complementary DNA strand through the transient nick [7]. Type II topoisomerases are composed of two identical subunits of 170 or 180 g/mole. They require ATP hydrolysis for catalytic activity and can alter DNA topology by creating transient double strand breaks, through which a second intact double helix is passed [9]. Mammals possess two isoforms of type II topoisomerases, α and β, which are encoded by separate genes [13–15]. All cells contain type I and type II topoisomerases. However, only the type II enzymes seem to be essential for cell survival, whereas the type I enzymes can be complemented by the type II enzymes.

Enzymes such as topoisomerases, which generate breaks in the DNA, carry a high risk of causing genomic disorders. Under normal circumstances, this unfavourable property of topoisomerases does not come into effect, because the critical strand-passing step is only a very short-lived catalytic intermediate. However, conditions that significantly increase the half-lives of topoisomerase-linked DNA intermediates induce a number of DNA disorders including mutations, insertions, deletions and chromosomal aberrations [9], which in summary are deleterious to the cell [16,17]. A number of powerful anti-cancer drugs act by stabilizing the covalent topoisomerase–DNA intermediates [18,19], thereby converting topoisomerases into cell poisons [20,21]. The efficacy of these drugs is closely related to the cellular levels and activities of topoisomerases [22–24] and to the responsiveness of the enzymes to the drugs. Thus, down-regulation of topoisomerases [25,26], or mutations [27–30] and epigenetic modifications [31–33] that will produce topoisomerase-variants that are less susceptible to these drugs, result in resistance of the tumor to treatment. Consequently, in recent years, quantitative assessment of cellular levels of topoisomerases and detection of the molecular effects of topoisomerase poisons have become an objective of increasing interest to clinical oncologists [20,34–37].

In this paper we review state-of-the-art techniques for extracting and purifying topoisomerases from various tissues or cell cultures, and of separating isoenzymes and isoforms of topoisomerases. We summarize assays that are suitable for measuring the

catalytic activity of topoisomerases and for determining the molecular effects of topoisomerase poisons.

2. Purification of eukaryotic topoisomerases and separation of isoforms

2.1. Purification of eukaryotic type I topoisomerases

Topoisomerase I (at that time called protein ω) was purified from *Escherichia coli* for the first time in 1971 by Jim Wang [38]. The basic preparative strategy of this first purification, consisting of salt extraction of the cells, removal of contaminating DNA, ammonium sulfate precipitation of the enzyme, renaturation and purification by several steps of ion exchange- and/or affinity chromatography on DNA-like matrices (see Fig. 1) has since been conserved through all protocols published on the purification of eukaryotic topoisomerase I from HeLa cells [39], avian erythrocytes [40–42], *Drosophila melanogaster* [43–45], *Saccharomyces cerevisiae* [46], calf thymus [47–49] and mouse mammary carcinoma FM3A cells [50]. The following paragraph will compile the essence of these purification protocols. In addition, our current protocol for large scale purification of human topoisomerase I expressed in yeast is described in more detail.

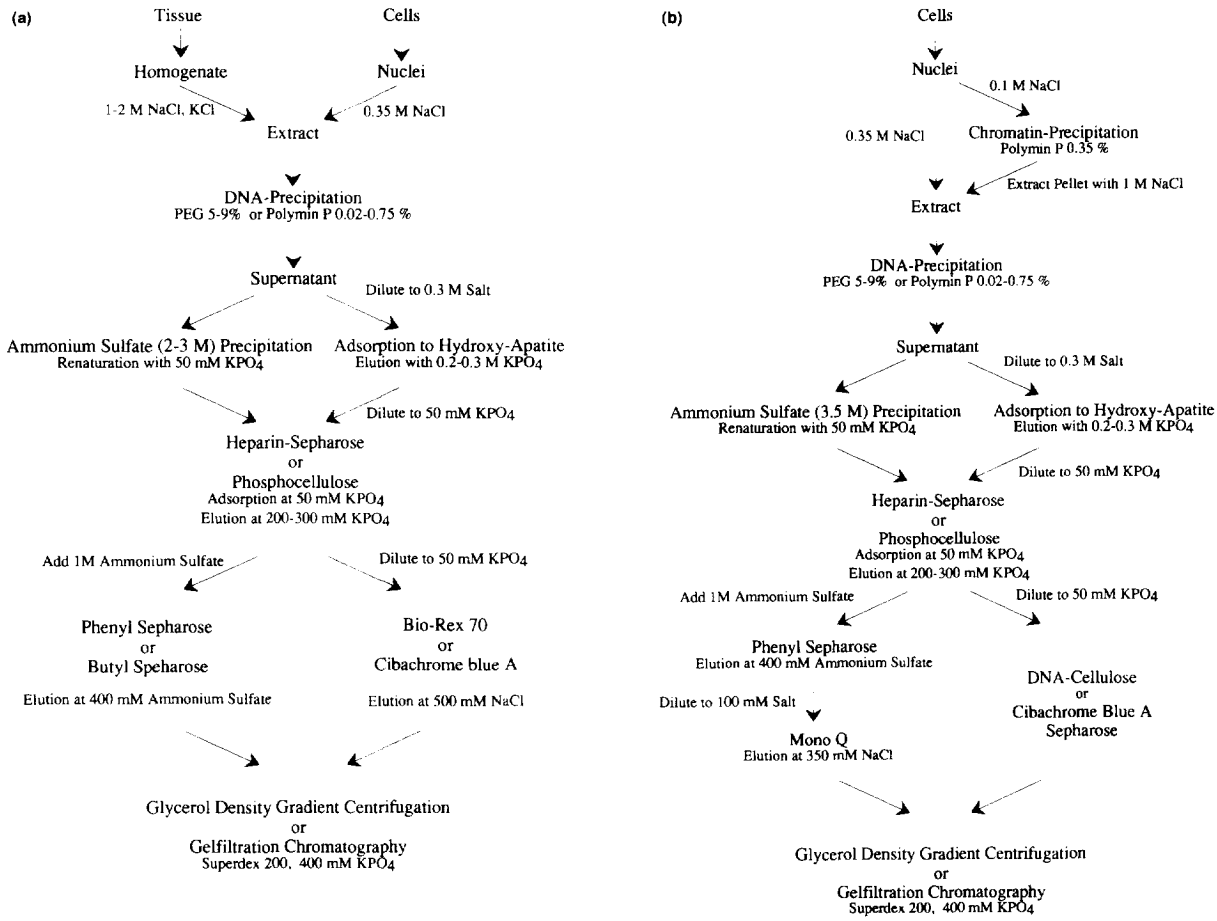

Fig. 1. (a) Strategies for purifying eukaryotic type I topoisomerases. (b) Strategies for purifying eukaryotic type II topoisomerases.

2.1.1. General guidelines

The amount of effort needed for the chromatographic phase of purification is inversely related to the degree of purification obtained during the pre-chromatography stages. Optimization of these steps seems to be at least as essential as devising an optimal chromatographic strategy later on. Topoisomerase I is a monomeric enzyme of 100 g/mole. However, it is subject to rapid aminoterminal proteolysis during the early preparative steps, which once lead to the belief [51] that two forms of the enzyme, with molecular masses of 68 and 100 g/mole in-vivo, may exist. The proteolytic 68 g/mole fragment of topoisomerase I is catalytically active, but lacks regulatory domains [47]. In order to avoid proteolysis, the early purification steps should be carried out rapidly, on ice, and in the presence of effective serine- and metallo-protease inhibitors. Moreover, topoisomerase I needs to be phosphorylated on the aminoterminal domain, in order to be fully functional [32,52–54]. Usually, a strong phosphatase activity copurifies during most preparative steps. Thus, the enzyme rapidly looses activity during purification by dephosphorylation, unless potent phosphatase inhibitors are included. It should be noted, however, that some of the most potent phosphatase inhibitors, such as NaF, inhibit enzyme activity. Topoisomerase I should not be exposed to pH values higher than the p*I* value, which is 7.9. Most commonly, the pH is kept between 7.0 and 7.4 during all preparative steps. Like all nuclear proteins, topoisomerase I needs to be kept in the presence of thio-reductive compounds such as β-mercaptoethanol or dithiotreitol, in order to prevent formation of aberrant intramolecular cysteine bonds.

2.1.2. Isolation of nuclei

When purifying topoisomerase I from cultured, or other single cells of higher eukaryotes, purification of cell nuclei (as described in some detail in [55,56]) is an essential first step. It comprises cell lysis with non-ionic detergents, such as Triton X-100, followed by sedimentation across a single-step gradient of 30% sucrose. Care needs to be taken that the isolated nuclei, which can be collected from the pellet underneath the sucrose cushion, become neither damaged nor contaminated by non-lysed cells, which will sediment as well. Various cell types differ in the amount of detergent and the time of treatment needed for an optimal result.

2.1.3. Salt extraction

In the case of bacteria, yeast and tissues whose nuclei cannot be isolated first, extraction of topoisomerase I from homogenates is usually obtained using 0.5–2 *M* concentrations of NaCl or KCl. If isolated nuclei are to be extracted, 0.35 *M* concentrations of NaCl are sufficient. Under these conditions, DNA-modifying enzymes will be extracted almost selectively, whereas histones and other structural proteins of the nuclear scaffold will remain bound to the genomic DNA [40,42].

2.1.4. Removal of DNA

Fragments of genomic DNA are always extracted together with topoisomerase I. These fragments will bind to the enzyme at later purification steps, when the ionic strength is lowered. They can link topoisomerase I to other DNA-binding proteins present in the extract. These protein–DNA complexes may become insoluble and are also difficult to separate later. Finally, contaminating genomic DNA will act as a competitive inhibitor in catalytic assays of topoisomerase I. Therefore, DNA contaminations need to be removed as early as possible. Several DNA precipitation procedures, which do not precipitate or inactivate topoisomerase I, have been established: Streptomycin [38], poly(ethylene glycol) (PEG 6000–8000, 5–9%) [45,49,51] and polyethylene imine (polymine P, 0.02–0.75%) [40,41]. It should be noted that small DNA fragments in particular will take considerable time for precipitation. This is most notable with PEG, taking up to 12 h for complete precipitation [4–9]. Alternatively, filtration with glass fiber filters (Whatman GF/A) [42] or adsorption to hydroxyapatite have been employed for removing DNA. The latter procedure has also been used in conjunction with DNA precipitation in many protocols as a means of concentrating the extracts. Both DNA and topoisomerase will bind to hydroxyapatite at NaCl concentrations up to 0.3 *M*, but topoisomerase can be eluted at 0.4–0.5 *M* potassium phosphate, whereas DNA will bind much more tightly [48,49].

Ammonium sulfate precipitation is an easier way to concentrate the nuclear extract before chromatog-

raphy. Topoisomerase I can be reversibly denatured and precipitated by 2–2.5 *M* ammonium sulfate, whereas topoisomerase II stays soluble for the most part at these concentrations, making a crude separation of the two types of topoisomerases possible [49]. In the presence of 1 *M* ammonium sulfate, topoisomerase I appears to be more stable.

2.1.5. Chromatography

The final purification stage usually comprises two chromatographic steps. The first is a mixed interaction chromatography on DNA-like matrices, carrying strong polyanion ligands, such as heparin Sepharose [49,50] or phosphocellulose [38,40,45,51]. The enzyme binds to these matrices at low salt concentrations (<100 m*M* NaCl or KCl or <50 m*M* potassium phosphate) and can be eluted by 0.5–0.6 *M* NaCl or 0.2–0.3 *M* potassium phosphate. For the second chromatographic step, cation-exchange chromatography (BioRex 70) [49], DNA affinity chromatography [51], affinity chromatography with immobilized Cibachrome blue A [41] or hydophobic interaction chromatography [40,48,50] have likewise been used. The latter procedure is preferred in more recent publications [47,48]. As the eluents from the previous chromatographic step can be adsorbed to the hydrophobic matrix without further dilution or desalting, this procedure is also less time-consuming. Phenyl Sepharose is the hydrophobic matrix most widely used. However, we have made the observation that butyl Sepharose gives the best results. Topoisomerase I will bind to both hydrophobic matrices in the presence of 0.8–1 *M* ammonium sulfate and can be eluted by lowering the concentration to 0.4–0.6 *M* ammonium sulfate [40,48,50]. Finally, it should be noted that some authors attach value to further fractionating the enzyme preparation after the final chromatographic step by glycerol density gradient centrifugation, in order to remove enzymatically active proteolytic fragments [43,47].

2.1.6. Purification of human topoisomerase I overexpressed in yeast

Obtaining pure enzyme in large amounts has often been the limiting step in studies of eukaryotic topoisomerases. However, inducible overexpression of human DNA topoisomerase I in *Saccharomyces cerevisiae* cells lacking endogenous topoisomerase I followed by a simple purification procedure has made it possible to obtain milligrams of pure wild type or mutant enzymes from 10 l of culture. Placing the coding sequences of the gene TOPI downstream of an inducible promotor PGAL1 on a multicopy plasmid makes it possible to direct synthesis of foreign topoisomerase I in yeast [57]. The PGAL1 promoter has the advantage that its transcription is well regulated by carbohydrate sources in the medium, which is important as continuous overexpression of topoisomerase I is deleterious. Expression of topoisomerase I can be induced by addition of 2% galactose to cultures grown to mid-log phase in a glucose-free medium. Induction should be carried out for an additional 6–12 h before harvesting the cells by centrifugation. Cell disruption can be performed using liquid nitrogen or glass beads [58] followed by extraction of topoisomerase I in a buffer containing at least 0.5 *M* NaCl. Our studies show that the best purification of human topoisomerase I overexpressed in yeast is obtained by initial precipitation of the enzyme in 3 *M* ammonium sulfate followed by two or three chromatographic steps. After gentle renaturation of the enzyme, the extract is applied to a heparin Sepharose matrix in a buffer containing 100 m*M* NaCl. Topoisomerase I is eluted from this matrix by 0.5–0.7 *M* NaCl. Elution can be performed either by step elution or a linear gradient between 0.4 and 1 *M* NaCl. The eluent can be concentrated on a second heparin Sepharose column or can be applied directly to a phenyl- or butyl Sepharose matrix. For binding of topoisomerase I to the hydrophobic matrices, the salt concentration of the eluent should be adjusted to 1.2 *M* ammonium sulfate. Elution can be obtained with a linear gradient of 1.0–0.2 *M* ammonium sulfate. Alternatively, hexahistidine-tagged topoisomerase I expressed in yeast, as described above, has been purified by nickel chelate affinity chromatography [59].

2.2. Purification of type II topoisomerases

Eukaryotic topoisomerase II differs structurally from the bacterial type II enzyme, gyrase, which is a tetrameric complex composed of pairs of two different subunits, gyrA and gyrB, whereas eukaryotic topoisomerase II enzymes are homodimeric proteins

composed of identical monomers which combines the functions of gyrA and gyrB. In mammals there are two structurally closely related isoforms of topoisomerase II, designated α and β, with the monomeric sizes of 170 (α) and 180 g/mole (β), respectively [60,61]. Due to the close structural similarity between these two isoenzymes, they copurify and special efforts have to be made to separate them. Recently it has been discovered that α/β-heterodimers of topoisomerase II may exist in low quantities in HeLa cells (personal communication, Harald Biersack, Dept. Mol. Struc. Biol., Univ. Åarhus, Denmark).

Eukaryotic topoisomerase II has been purified from calf thymus [62,63], *Drosophila melanogaster* [43,44], various cultured tumor cell lines [56,60,64] and from the yeast *Saccharomyces cerevisia* [64]. Compared to topoisomerase I, purification is more difficult, because topoisomerase II levels are at least ten times lower in the cells. Moreover, the expression of topoisomerase II is proliferation-dependent and alters during the cell cycle [39,66], which means that not all tissues are equally suitable sources for purification of the enzyme. Strategies have been established to purify topoisomerase I and topoisomerase II simultaneously [48]. However, in view of the different physico-chemical properties of the two enzymes, it seems more advisable to use dedicated protocols. The general purification strategy is summarized in Fig. 1. The p*I* of topoisomerase IIα is between pH 5 and pH 6 [67,68], whereas the p*I* of topoisomerase IIβ is about pH 4.5 (unpublished observation). However, both isoenzymes are most active and stable between pH 7.5 and 8 [67] which is why the pH should be kept at these values during all preparative steps.

2.2.1. Pre-chomatographic steps

Most pre-chomatographic steps are essentially the same for topoisomerases I and II. As these have already been described above, this section will focus on the differences in the techniques used for preparation of topoisomerase II compared to topoisomerase I.

There are two different strategies for extracting topoisomerase II from isolated nuclei: The most common one is very similar to that of topoisomerase I, with the exception that a higher pH (7.5–8) should be maintained throughout the procedure. The other strategy involves lysis of the isolated cell nuclei at salt concentrations that will not dissociate the enzyme from the DNA (e.g. 100 m*M* NaCl). DNA-bound enzyme is then precipitated by 0.35% polyethylene imine [48,63] or PEG [69] and subsequently extracted from the chromatin by 0.55 *M* NaCl. Precipitation of contaminating DNA has to follow in both extraction protocols. As already mentioned, topoisomerase II needs higher concentrations (3–3.5 *M*) of ammonium sulfate for precipitation than topoisomerase I [70]. Alternatively, or in addition, adsorption to hydroxyapatite has been used for concentrating the crude extract [43,48,64].

2.2.2. Chromatography

Strategies of chromatographic purification are very similar to those described for topoisomerase I. However, three steps are usually required for purification to homogeneity: (i) Heparin Sepharose [70] or phosphocellulose [43,64] are used as the first chromatographic step; (ii) Affinity chromatography with DNA cellulose [64] or Cibachrome blue A agarose [62,63] serve as the major purification steps. It should be noted that, due to its ATPase domain, topoisomerase II binds almost irreversibly to Cibachrome blue A. Thus, a high purification factor has to be paid for by low yields and extremely high salt concentrations (2–3 *M* NaCl) in the eluent. In more recent publications, a combination of automated hydrophobic interaction and anion-exchange chromatography has been preferred [48,60,61]; (iii) Final purification is usually obtained by gel permeation chromatography [64] and/or glycerol density gradient centrifugation [43].

2.2.3. Large scale purification of eukaryotic topoisomerase II

Because of the low cellular levels of topoisomerase II, large scale purification of this enzyme has been laborious in most cases. The difficulty of obtaining pure topoisomerase II in large amounts has in many cases hampered the use of this enzyme in mechanistic studies. However, like topoisomerase I, eukaryotic topoisomerase II can be overexpressed in yeast by employing the PGAL1 promoter and can be purified in large quantities from this organism. Expression and purification of topoisomerase II from

the yeast extract has been described by Worland and Wang [65]. More recently, full-length or parts of topoisomerase II have been overexpressed in yeast *Saccharomyces cerevisiae* or in *Escherichia coli* as hexahistidine-tagged fusion proteins and purified by nickel chelate chromatography [71,72].

2.3. Separation of isoforms and epigenetic variants

For most applications and questions it is not necessary to purify topoisomerases to homogeneity. Nevertheless, it may be important to differentiate between topoisomerase I and topoisomerase II, and to discriminate between the two mammalian isoenzymes of topoisomerase II, or even between epigenetic variants of these isoenzymes. Some of these aims can be reached by using specific assays, as will be discussed in the following chapter. In most cases, however, it will be necessary to physically separate these various enzymes before measuring their activity by non-specific assays.

2.3.1. Techniques for separating topoisomerase I from topoisomerase II

In most cases it is not necessary to separate the two enzymes physically as there are type-specific assays (see Section 3). Physical separation of topoisomerase I from topoisomerase II can be obtained in three ways: (1) Fractionated precipitation with 2 *M* ammonium sulfate (topoisomerase II), followed by 3.5 *M* ammonium sulfate (topoisomerase I); (2) adsorption to a strong anion-exchanger such as MonoQ, at pH 8. Topoisomerase I will not bind, whereas topoisomerase II can be eluted with 0.4 *M* NaCl; and (3) gel permeation chromatography: Superdex 200 is the separation medium that gives the best results. Separation should be carried out in the presence of at least 400 m*M* potassium phosphate in order to avoid adsorption of the enzyme to the matrix. Whereas topoisomerase II will elute as a dimer with an apparent size of 340–360 g/mole, topoisomerase I usually elutes as a monomer with a molecular size of 120–150 g/mole [56].

2.3.2. Techniques for the separation of topoisomerase IIα from topoisomerase IIβ

There seems to be a difference in the pH optimum of the catalytic activity of topoisomerase IIα and IIβ [73]. However, this observation has not been validated on a broad basis. It can probably not be used for a clear-cut differentiation between the two isoenzymes in all cases. Separation of topoisomerase IIα from IIβ by chromatographic methods is difficult due to the high degree of structural similarity between these two isoenzymes. Separation has been achieved by eluting the two enzymes bound to MonoQ by a shallow NaCl gradient [60,61,67,73]. Topoisomerase IIα will elute at slightly lower salt concentrations than topoisomerase IIβ. Unfortunately, there are variants of topoisomerase IIα, which will bind more tightly to anion-exchange resins and coelute with topoisomerase IIβ [67,73]. Recently, we have obtained isoenzyme-specific peptide antibodies directed against topoisomerase IIα or IIβ [39] which can be used for immunoprecipitation techniques. Finally, topoisomerase IIβ alone can be prepared from cells that do not express the α-isoenzyme, such as confluent 3T3-fibroblasts [74].

2.3.3. Resolving epigenetic variants of topoisomerase I and topoisomerase II

As already mentioned, the proteolytic 68 g/mole fragment of topoisomerase I can be removed by glycerol density gradient centrifugation or by gel permeation chromatography. There are several variants of 170 g/mole topoisomerase IIα, which differ in p*I* and catalytical properties, and which most probably represent differently phosphorylated states of the enzyme [67,68,73]. These variants can be separated by anion-exchange chromatography or, more efficiently, by chromatofocussing, using a weak anion-exchanger, such as MonoP, and mixtures of ampholytic buffer substances for isocratic elution [56,67]. Several isoactivities with p*I* values of between 5 and 6.5 have been resolved by this procedure.

3. Assessment of DNA topology and DNA topoisomerase activity

3.1. Cell-free assays for topoisomerase activity

Topoisomerases function by altering the topological state of the DNA. Fig. 2 summarizes different types of topological changes of the DNA that can be

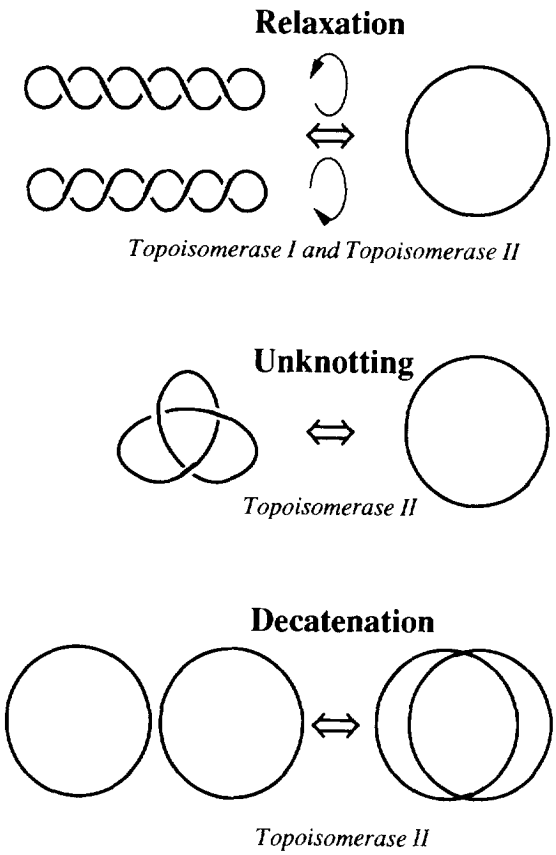

Fig. 2. DNA topoisomerizations catalyzed by type I and type II DNA topoisomerases.

catalyzed by these enzymes in principle. Topoisomerase I cleaves and religates only one strand of the double helix and allows passage of the complementary strand through the transient nick, thereby altering the pitch of the DNA. Thus, it can release positive or negative supercoils from closed-circular DNA plasmids [38]. Topoisomerase I can not catalyze more complex changes in DNA topology, such as catenation/decatenation or knotting/unknotting, which require the transient insertion of DNA double strand breaks and the passage of an intact second double helix [10], unless very high concentrations of the enzyme are present with adjacent nicks being formed concurrently close to each other, by two independent enzyme molecules [75,76]. Under most conditions, these more complex DNA topoisomerization reactions are reserved to topoisomerase II, which simultaneously inserts a transient break into both DNA strands and passes another intact double helix through the gap. Topoisomerase II also relaxes supercoiled closed circular DNA-plasmids [44], but it does so less avidly than topoisomerase I. Moreover, it requires ATP for the reaction, whereas topoisomerase I acts independently of free metabolic energy [10]. The techniques for measuring topoisomerase activity have been reviewed five years ago [77]. The following section will summarize these older techniques and give an update on more recent developments.

3.1.1. Catalytic assays for DNA topoisomerization

Several naturally occuring DNA structures have been found, which are able to form more than one unique topoisomer that can be demonstrated by biochemical methods. These DNA structures can be multiplied in-vivo and serve as substrates for simple catalytic assays of DNA topoisomerases. The most widely used topoisomerization assay is relaxation of supercoiled plasmid DNA. For this purpose, several plasmids have been used [38,44], the most common being pBR 322, which contains several strong cleavage sites for both types of topoisomerases. Plasmid relaxation can be used as a topoisomerase I specific assay by omitting ATP from the reaction medium, or, more safely, by adding the ATPase-poison, orthovanadate, which inhibits most forms of topoisomerase II [56,61,67,73,78], but not topoisomerase I. The various plasmid DNA topoisomers formed by the enzymes can be easily demonstrated as a "DNA-ladder" by analysis on a 1% agarose gel (see Fig. 3). Electrophoresis should be carried out in the absence of intercalators such as ethidium bromide, as this will change the topology of the closed circular plasmids. It is likewise important to avoid heating the gel during electrophoresis. More complicated procedures have been devised for the analysis of plasmid topology [79,80]. However, these can only be employed with highly purified enzyme preparations and do not bear any significant advantage over agarose chromatography. Enzyme activity can be titrated by measuring plasmid DNA relaxation by serial dilutions of the enzyme preparation. One unit of enzyme activity is usually defined as the amount of enzyme that will completely relax 250 ng of pBR 322 DNA at 37°C within 30 min. Specific relaxation activity of

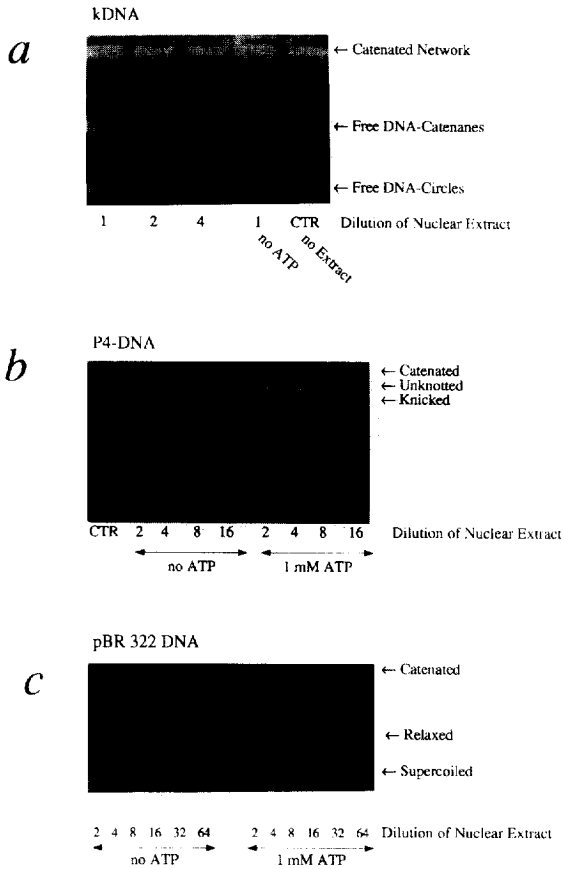

Fig. 3. Catalytical assays for topoisomerases: Crude nuclear extract of human A431 epidermoid cells was obtained by extracting 2×10^7 isolated nuclei with 0.35 *M* NaCl. The extract was prediluted with reaction buffer (10 m*M* bis–Tris–propane, pH 7.9, containing 10 m*M* $MgCl_2$, 100 m*M* KCl and 0.1 m*M* DTT), as indicated. A 2-μl volume of prediluted extract was reacted with the respective DNA substrate in the absence or presence of 1 m*M* ATP in a final volume of 30 μl of reaction buffer. Controls were without extract. Incubation at 37°C for 30 min was terminated by addition of 1% SDS. Samples were then digested with 1 mg/ml of proteinase K at 37°C for 30 min. Gel electrophoresis was performed at 1 V/cm for 24 h in 1% agarose gels with TAE-buffer. Following electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide. Fluorescence of ethidium bromide in the gels (exitation 302 nm, emission >600 nm) was documented by Polaroid photography. (a) Decatenation: 200 ng of *Crithidia fasciculata* catenated network kinetoplast DNA (kDNA) were used as a substrate. (b) Unknotting: 200 ng of knotted P4-plasmid DNA were used as a substrate. (c) Relaxation: 250 ng of supercoiled pBR 322 plasmid DNA were used as a substrate.

topoisomerase I is 10^5–10^6 units/mg protein. Specific relaxation activity of topoisomerase II is at least one order of magnitude lower. Catenation (see Fig. 3) or knotting [81] of plasmid DNA in the presence of ATP is an indication of topoisomerase II activity being present, as topoisomerase I cannot catalyze these reactions. However, simultaneously occuring relaxation makes interpretation of catenation or knotting difficult in quantitative terms. Naturally occuring DNA molecules of more complex topology, commonly used for specifically measuring the activity of topoisomerase II, are the knotted DNA of the bacterial phage P4 (P4-DNA) [51,82,83] and the catenated network DNA (k-DNA) from the kinetoplast of Trypanosoma species or from *Crithidia fasciculata* [64,84]. Both DNA substrates can be obtained commercially. Knotted P4-DNA migrates as a broad smeary band in agarose gel electrophoresis. Due to topological constraints, it hardly incorporates any ethidium bromide. P4-unknotting can be visualized as the appearance of two sharp, brightly stained DNA bands, representing relaxed and nicked forms of the P4-DNA plasmid (Fig. 3). P4-unknotting is the test most suitable for titrating topoisomerase II activity, if plasmid relaxation can not be used, because topoisomerase I is also present. One P4-unknotting unit has been defined as the amount of topoisomerase II that will completely unknot 100 ng of P4-DNA in the presence of 0.5–1 m*M* ATP at 37°C within 30 min [83]. Specific P4-unknotting activity is slightly lower than relaxation activity. Decatenation of k-DNA is also a specific assay for topoisomerase II catalytic activity, but is less sensitive than P4-unknotting. As the high molecular catenated network of k-DNA is too large to enter an agarose gel, topoisomerase II-specific decatenation can be monitored by the release of mobile DNA catenanes and circles from the immobile network (Fig. 3). Alternatively, k-DNA can be pelleted by centrifugation, whereas released DNA circles will stay in solution and, thus, can be measured fluorometrically in the supernatant [85].

3.1.2. Cell-free assays for topoisomerase-directed drug effects

Some of the most powerful therapeutic substances for the systemic treatment of cancer used today are inhibitors of DNA topoisomerases. There are two

types of inhibitors with different mechanisms of action. The first group of substances, termed topoisomerase poisons, includes antracyclines, aminoanthracenes, podophyllotoxins, aminoacridines, ellipticines, quinolones and flavonoids acting on topoisomerase II [20,21,35,86,87] and camptothecins acting on topoisomerase I [18,88–90]. These compounds stabilize the covalent DNA-topoisomerase intermediate by stimulating the cleavage reaction and/or inhibiting the religation step. The second group of substances, termed topoisomerase inhibitors, such as aclarubicine [91], β-lapachone [92], chebulaic acid [93] and certain synthetic flavonoids [94], inhibit the non-covalent DNA binding or the cleavage reaction of topoisomerases. For the simultaneous screening of topoisomerase I-targeted drug effects, both of the poison- and of the inhibitor-type, a strategy has been proposed [93], which is based on the alteration of the electrophoretic mobility of pBR 322 plasmid DNA, by the combined action of topoisomerase I and inhibiting drugs. As shown in Fig. 4, the mobility of the naturally supercoiled closed circular double stranded plasmid DNA increases upon topoisomerase I-mediated relaxation, if electrophoresed in a 1% agarose gel with 0.5 μg/ml of ethidium bromide. This change in mobility of the topoisomers is caused by interaction of the DNA with ethidium bromide leading to the introduction of positive supercoils in closed circular DNA molecules. In the presence of topoisomerase I and camptothecin, which binds to the covalent DNA-topoisomerase I intermediate and inhibits the religation half-reaction [18], topoisomerase I introduces nicks into one of the DNA strands. After proteinase K-digestion of the covalently attached enzyme, the resulting open-circular plasmid migrates in a similar position as DNase I-nicked pBR 322 [95]. The migration of the open-circular plasmids is unaffected by intercalation with ethidium bromide and is slower than for closed-circular and linearized plasmid forms. In contrast, substances such as β-lapachone, or EMD 50 689 [94], which inhibit the catalytic activity of the enzyme but do not stabilize the covalent DNA intermediate, will inhibit plasmid relaxation but will not induce nicked plasmid forms. A similar result is obtained with topoisomerase II and topoisomerase II-directed drugs, with the exception that this enzyme will linearize the plasmid, because it simultaneously cleaves both DNA strands.

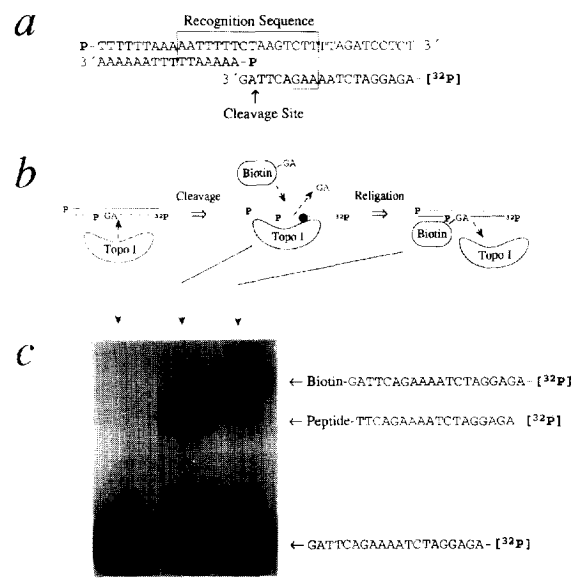

Fig. 4. Topoisomerase-mediated relaxation, nicking and linearization of a plasmid DNA. Procedure: 250 ng pBR 322 plasmid DNA were incubated with 200 U of human topoisomerase I or II in the absence or presence of camptothecin, etoposide or EMD 50 689. The control was pBR 322 DNA without drugs and without enzymes. Linearized pBR 322 was obtained by digestion of 250 ng DNA with 40 units of EcoRI endonuclease. Nicked pBR 322 DNA was obtained by digestion of 1 μg of DNA with 0.2 u of DNase I in the presence of 0.25 μg/μl ethidium bromide, 20 m*M* $MgCl_2$, 0.2 m*M* DTT, 10 m*M* bis–Tris–propane, pH 7.9, at 37°C for 5 min (modified according to [133]). The assay had a final volume of 40 μl of reaction buffer (10 m*M* bis–Tris–propane, pH 7.9, containing 10 m*M* $MgCl_2$, 10 m*M* KCl, 0.1 m*M* DTT and 10% dimethylsulfoxide). Incubation at 37°C for 30 min was terminated by addition of 1% SDS. Samples were then digested with 1 mg/ml of proteinase K at 37°C for 30 min. Gel electrophoresis was performed at 0.4 V/cm for 12 h in 1% agarose gels with TBE-buffer containing 0.5 μg/ml of ethidium bromide. Fluorescence of ethidium bromide in the gels (exitation 302 nm, emission >600 nm) was documented by Polaroid photography. Interpretation: The migration distance of naturally supercoiled pBR 322 DNA (lane 3) becomes increased upon relaxation by topoisomerase I (lane 7) or topoisomerase II (lane 8), when subjected to 1% agarose gel electrophoresis in the presence of ethidium bromide, because the relaxed plasmid can incorporate more of the intercalator and as a result becomes more positively supercoiled. Linearization (lane 1) or nicking (lane 2) result in forms of the plasmid migrating more slowly, because these will not be supercoiled by ethidium bromide any more. It can clearly be seen (lanes 4 and 5), that in the presence of topoisomerase I, camptothecin, a topoisomerase I poison, induces the nicked plasmid form in a dose-dependent manner, whereas there is no effect in the absence of the enzyme (not shown). In contrast, EMD 50 689, a synthetic flavonoid recently shown [98] to inhibit topoisomerase I catalytic activity, inhibits topoisomerase I-mediated pBR 322 relaxation but does not induce nicking (lane 6). The topoisomerase II poison, etoposide, induces linearization of pBR 322 DNA in the presence of topoisomerase II (lane 9).

The linearized plasmid form migrates slightly further into the gel than the nicked from. Thus, inhibition of relaxation without linearization or nicking indicates the action of a topoisomerase inhibitor, whereas formation of open-circular or linearized plasmid DNA can be taken as a measure of the effects of topoisomerase poisons. Since the formation of nicked or linearized plasmid forms by topoisomerase I or II, respectively, is a stoichiometric process, it is necessary to add sufficient amounts of enzyme to the assay. However, with increasing amounts of enzyme, background cleavage of the DNA substrate in the absence of drug will also rise. Thus, optimization of the enzyme-to-DNA ratio in the assay is crucial. The best results will be obtained with 100–200 units of topoisomerase per 250 ng of pBR 322 DNA in a final volume of 20–30 μl.

3.2. Dissecting the catalytic cycle

Topoisomerization of complex DNA substrates, such as plasmids, is the result of repeated rounds of the complete sequence of DNA cleavage, strand passage and DNA religation reactions. In order to analyze these part-reactions separately, oligonucleotide suicide-substrates of topoisomerase I [96,97] and topoisomerase II [19,91] have been designed, which restrict the enzyme to a single round of cleavage and religation and allow for addressing these two half-reactions separately. The function of these non-catalytic assays is exemplified by the oligonucleotide suicide-substrate assay of topoisomerase I: A double-stranded 36-mer oligonucleotide containing a strong topoisomerase I cleavage sequence [98] is composed, as shown in Fig. 5a. The strand to be cleaved has a nick two base pairs 3′ to the major cleavage position and is ^{32}P-labelled at the 5′-end. All other 5′-ends, including that of the nick, are blocked by phosphorylation, in order to prevent religation at these sites. The oligonucleotide is mainly cleaved in a position two base pairs 5′ to the nick [96,99]. The dinucleotide which is cleaved off escapes from a further religation reaction by diffusion and religation to the 5′-end distal to the nick is blocked by phosphorylation. Therefore, cleavage is irreversible and the substrate becomes quantitatively converted to a covalent complex with the enzyme (Fig. 5b). For religation, a 1000-fold excess of a 3′-biotinylated GA-dinucleotide can subsequently be added to the

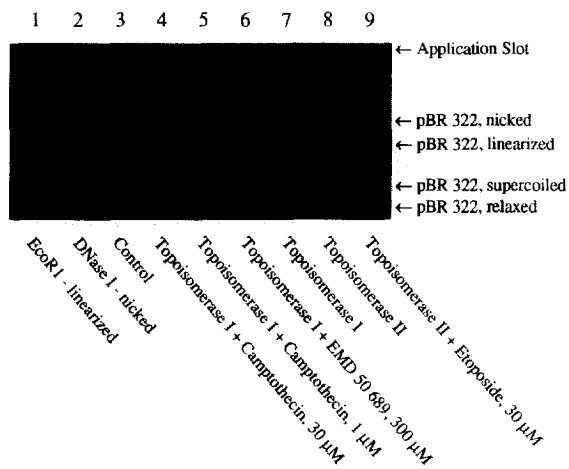

Fig. 5. Separate measurement of topoisomerase I-mediated cleavage and religation: (a) The oligonucleotide substrate is composed of a 36-mer non-cleaved strand, a 20-mer cleaved strand and a 16-mer duplex-forming strand. Non-cleaved and duplex-forming strands were nonradioactive, the cleaved strand was radioactively phosphorylated on the 5′-ends with *E. coli* T4 polynucleotide kinase. The oligonucleotides were hybridized in a ratio of 1:1.5:2 (cleaved– non-cleaved–duplex-forming). (b) Suicide cleavage of the oligonucleotide was initiated by addition of 200 units of purified human topoisomerase I per 0.2 pmoles of substrate and was allowed to continue for 30 min at 30°C. Religation was subsequently started by addition of 200 pmoles of the biotinylated dinucleotide and was carried out in the presence of 330 m*M* NaCl at 37°C for 30–60 min. (c) Electrophoresis of the non-cleaved (left), the cleaved (middle) and the religated substrate (right) in a 14% polyacrylamide gel under denaturing conditions. Samples were trypsinized and denatured with formamide and heating (96°C for 5 min) before electrophoresis.

topoisomerase I–DNA complex as a religation substrate together with 330 m*M* NaCl, in order to prevent recleavage of the religated DNA–biotin adduct (Fig. 5b). After ethanol precipitation and trypsinization, non-cleaved, cleaved and religated forms of the labelled oligonucleotide strand can be electrophoretically separated on 0.5 mm 14% polyacrylamide gels under denaturing conditions and visualized by autoradiography (Fig. 5c). Cleaved and religated forms of the labelled oligonucleotide strand will migrate retardedly, because they are covalently linked to a protease-resistant peptide of topoisomerase I or biotin, respectively. Alternatively, for a quantitative assessment of the religation reaction, the fraction of the cleaved oligonucleotide strand religated to the biotinylated dinucleotide can be captured

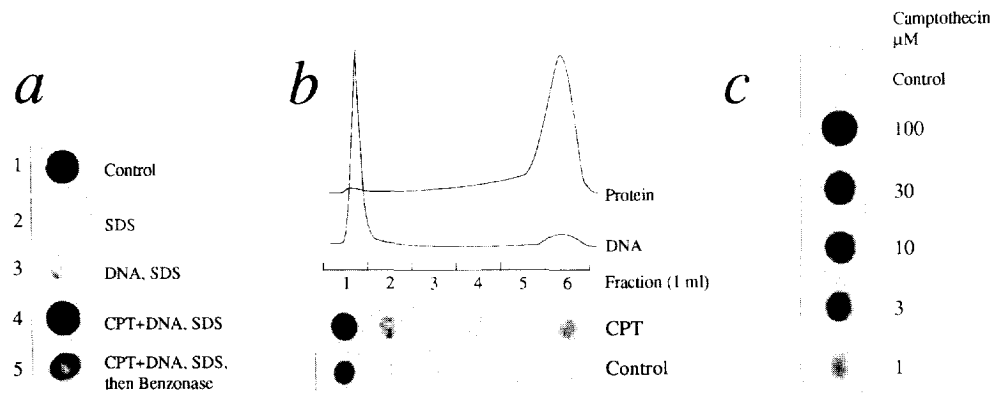

Fig. 6. Detection of covalent topoisomerase–DNA complexes by immuno-dot-blotting: (a) Procedure: Immuno-dot-blotting of DNA-linked topoisomerase I: 400 u of human topoisomerase I were preincubated with 3 μg of calf thymus DNA in the presence of 2 m*M* $MgCl_2$ in a final volume of 500 μl of reaction buffer (10 m*M* bis–Tris–propane, pH 7.9, containing 10 m*M* $MgCl_2$, 10 m*M* KCl, 0.1 m*M* DTT and 10% dimethylsulfoxide). Incubation at 37°C for 30 min with (lines 4 and 5) or without (line 3) 30 μ*M* camptothecin. The reaction was terminated by addition of 0.2% SDS. Controls were: Untreated enzyme (line 1), SDS-treated enzyme not preincubated with DNA (line 2), enzyme preincubated with DNA and 30 μ*M* camptothecin, followed by SDS-denaturation and subsequent treatment with 250 u of Benzonase (line 5). Samples were filtered through nitrocellulose filters using a 96-well vacuum dot-blot aparatus (Schleicher and Schüll, Germany), followed by two washes (500 μl) with 10 m*M* Tris–HCl, pH 7.5, 50 m*M* NaCl. Nitrocellulose filters were irradiated (254 nm, 2 min), dried at 20°C for 12 h, and finally subjected to immunostaining. Immunostaining was carried out using a mouse monoclonal antibody against human topoisomerase I (kindly supplied by Dr. Igor Bronstein, Engelhard Institute, Moscow, GUS), peroxidase-labelled goat-anti-mouse IgG and the ECL system. Interpretation: Nitrocellulose binding of non-denatured topoisomerase I (line 1) is completely blocked by SDS (line 2). It becomes completely restored upon preincubation with calf thymus DNA and camptothecin (line 4) but only marginally with DNA alone (line 3). Digestion with detergent resistant endonuclease (Benzonase) abolishes camptothecin-induced filter binding (line 5), showing that covalent DNA-linkage of the denatured enzyme is responsible for absorption to nitrocellulose. (b) Procedure: Enzyme was preincubated with DNA in the absence or presence of 30 μ*M* camptothecin (CPT), and denatured with 0.2% SDS [see (a)], followed by gel chromatography (Sepharose 4B, column 10×1 cm, equilibrated and run with 0.5 ml/min of reaction buffer, containing 0.1% SDS), in order to separate DNA-bound from free topoisomerase I. Fractions of 1 ml were collected and analysed by immuno-dot-blotting. The elution profiles of enzyme or DNA alone, respectively, were determined by light absorption at 280 nm (Protein) or fluorescence of bisbenzimide (0.01 μg/ml)-stained DNA, respectively. Interpretation: Gel chromatography proves that only enzyme coeluting with the high molecular DNA binds to nitrocellulose filters in the presence of SDS. Pretreatment with camptothecin specifically increases the filter binding of the DNA-linked, but not of the free enzyme fraction. Thus, filter binding of the SDS denatured enzyme can be used for demonstrating covalent DNA-linkage. (c) Procedure: Topoisomerase I was preincubated with DNA in the absence (control) or presence of various concentrations of camptothecin, followed by immuno-dot-blot analysis, as described in (a). Interpretation: Camptothecin increases the covalent DNA-linkage of topoisomerase I in a manner reciprocal to the concentration of the drug.

by immobilized avidin. Radioactivity, bound to the beads can be taken as a measure of religation. These assays have proven valuable tools in elucidating the mechanism of enzyme action, but they can only be used with high concentrations of pure enzymes and, therefore, are not suitable for all purposes.

3.2.1. Inhibition of part-reactions

Detailed information on the effects of various drugs on the DNA cleavage and religation reactions has been obtained by including these substances into oligonucleotide suicide assays [28,100]. Inhibition or stimulation of the cleavage and/or of the religation reactions can, thus, be studied separately and without influencing each other. We have used this approach recently for the characterization of various novel flavone inhibitors of eukaryotic topoisomerase I [94].

3.3. Assays for demonstration of covalent topoisomerase–DNA complexes

3.3.1. Detection of covalent cleavage complexes in cell-free assays

While analytical efforts approaching topoisomerase–DNA interactions from the DNA-side offer the opportunity of screening many substances at the same time without large experimental effort, they require the use of pure or partially pure enzyme

preparations, not contaminated with DNases. Moreover, these assays are not very sensitive with respect to enzyme concentrations, because linearization and nicking are stoichiometric processes, which consume the enzyme present in the assay. Approaches for detecting covalent DNA–topoisomerase intermediates on the protein-side are more suitable for the analysis of crude preparations with low levels of enzyme: K-SDS precipitation utilizes the fact that SDS micelles of proteins can be precipitated with potassium. Thus, after incubating labelled DNA with topoisomerases and subsequent SDS-denaturation, the DNA label will be detected in the potassium precipitate, if covalent enzyme–DNA complexes have been formed during the incubation [101]. However, this approach is not specific for topoisomerase in as much as it will also detect covalent complexes between DNA and other proteins. A more specific and highly sensitive assay for the formation of topoisomerase–DNA adducts is the immuno-dot-blot [94]. This procedure uses the selective binding of covalent topoisomerase–DNA complexes to nitrocellulose in the presence of SDS [93]. Thus, crude topoisomerase preparations can be incubated with a non-specified DNA sample such as calf thymus DNA in the presence or absence of drugs. Subsequently, the incubation mixture is treated with SDS and filtered through nitrocellulose by vacuum. As shown in Fig. 6a–b, only enzyme that is covalently linked to the DNA will bind to the filter and can be specifically detected by immunostaining, whereas enzyme that is not linked to DNA will not bind. Enhancement of filter-binding can, thus, be clearly assigned to the action of a topoisomerase poison. The selectivity of the assay regarding the DNA-linkage of the enzyme can easily be tested by treating the sample with a detergent-resistant endonuclease, such as Benzonase, prior to filtration, which will abolish the immuno signal (Fig. 6a). Since specific antibodies for topoisomerase I [47], topoisomerase IIα and topoisomerase IIβ [39] are now available, the same analytical approach can be used for all forms of topoisomerase.

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