Topoisomerase I is the predominant nuclear protein from avian erythrocytes that can be covalently linked to DNA

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Nuclear extracts of erythrocytes contain proteins which stably or possibly covalently bind to DNA. These proteins can be detected by an assay which was originally developed to quantify stable binding of topoisomerases to DNA [Trask, DiDonato & Muller (1984) *EMBO J.* 3, 671–676]. In this report, we show that the number of activities detected by this assay in crude extracts of nuclei is limited predominantly to various forms of topoisomerase I. One form, a 50 kDa protein, copurifies with histone H1. Western blotting experiments suggest that the 50 kDa topoisomerase exists in chromatin along with the 105 kDa form. In addition, the ratio between the high and low- M_r forms is relatively constant in erythrocytes and embryonic fibroblasts. These results imply that the multiple forms are not unique to one tissue setting.

Topoisomerases act by transiently breaking the phosphodiester backbone of DNA and resealing the free DNA ends (for recent reviews see Gellert, 1981; Liu, 1983). This cycle of breaking and resealing allows for DNA strand passage and alterations in DNA topology which may accompany central events of transcription and DNA replication. As a class of DNA-binding proteins, topoisomerases are distinguished by their ability to form stable, probably covalent, complexes with DNA during the normal course of the nicking and closing cycle (Gellert, 1981; Liu, 1983; Trask *et al.*, 1984; Trask & Muller, 1983). We have developed a simple and straight forward technique, called the SDS/ K^+ assay, to quantify the covalent binding of topoisomerases to DNA (Trask & Muller, 1983; Trask *et al.*, 1984). The assay is based upon the concept that a covalent DNA–enzyme intermediate is trapped when SDS is added to denature the enzyme while it is acting on the DNA substrate. Thus, the transient DNA nick becomes permanent and topoisomerase is covalently attached to DNA at the site of the break. Those DNA molecules with attached proteins are then precipitated by the addition of KCl; however, free DNA is not precipitated by this method. The SDS/ K^+ assay was developed

using purified topo I (Trask & Muller, 1983); however, we considered the possibility that additional activities (besides topoisomerases) might exist which respond to such an assay. Proteins bound to DNA by electrostatic or ionic bonding are not detected since SDS effectively dissociates these complexes (Muller, 1983); therefore the technique is specific in that only detergent-resistant protein–DNA complexes are detected.

This work was conducted to determine if proteins that respond to the SDS/ K^+ assay represent a widespread or diverse class of nuclear proteins. The SDS/ K^+ method was used as a functional assay to identify and purify these proteins and conventional topoisomerase assays (relaxation of supercoiled DNA) were carried out to establish their relationship to topoisomerases. The surprising result is that, in crude nuclear extracts, topo I is the predominant and most likely the only protein detected by the SDS/ K^+ assay. Thus, we define the conditions whereby the assay is specific for DNA topoisomerase I.

Materials and methods

The SDS/ K^+ assay

SDS/ K^+ assays were performed exactly as described previously, using the analytical method (Trask *et al.*, 1984). The DNA substrate was bacteriophage λ DNA (from commercial sources) which was nick-translated with [3 H]dTTP. The specific radioactivities were $(2\text{--}5) \times 10^6$ d.p.m./ μ g.

Abbreviations used: SDS, sodium dodecyl sulphate; SDS/ K^+ , the protein–DNA binding assay; topo I, topoisomerase I.

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Routinely, SDS/K⁺ assays contained 5 ng of the labelled substrate in a final volume of 0.1 ml.

Purification procedures

Purification of SDS/K⁺ activities was carried out as follows (all steps were performed at 4°C). Nuclei were prepared from 1 litre of chicken blood by a single freeze-thaw cycle followed by two washes with 5 pellet vol. of buffer A (10 mM-Tris/HCl, pH 7.9, 1 mM-EDTA, 4 mM-MgCl₂, 1 mM-phenylmethanesulphonyl fluoride). Most or all of the haemoglobin was washed out and the nuclei were resuspended in 5 pellet vol. of buffer B [50 mM-Tris/HCl, pH 7.9, 1 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride, 10% (v/v) glycerol, 50 mM-sodium bisulphite]. An equal volume of buffer B containing 0.8 M-NaCl was added and the nuclei were extracted for 1 h. Polymix-P was then added to 1% (v/v) and, after 15 min at 0–4°C, nucleic acids were removed by centrifugation at 10000g for 20 min. Saturated (NH₄)₂SO₄ was then added to 45% saturation and the proteins were collected by centrifugation (10000g, 20 min). The precipitate was resuspended in buffer B and the salt adjusted to 0.1 M-(NH₄)₂SO₄ by conductivity measurements. This fraction was applied to a column (2.5 cm × 6 cm) of phosphocellulose (Whatman P-11) equilibrated with buffer B containing 0.1 M-(NH₄)₂SO₄. The column was washed and developed at a flow rate of 8 ml/h with a linear gradient of 0.1–0.8 M-(NH₄)₂SO₄ in buffer B. Conductivity, absorbance and SDS/K⁺ activities were measured on selected fractions. Active fractions were pooled and applied to a hydroxyapatite column (1 cm × 4 cm) (Bio-Rad) at a flow rate of 7 ml/h. Proteins were eluted with a linear gradient of phosphate buffer from 0.1 to 0.8 M. Phosphate buffer corresponds to potassium phosphate of the indicated molarity, pH 7.2, containing 1 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride and 10% (v/v) glycerol. Active fractions were pooled and stored at –70°C.

Preparation of antibody

To prepare antisera, the 105 kDa form of topo I was purified as described previously (Trask & Muller, 1983) and used as antigen. New Zealand white rabbits were injected subcutaneously with 150 µg of the antigen in Freund's complete adjuvant and boosted with 75 µg of antigen in Freund's incomplete adjuvant periodically. Radial diffusion assays in 1% agarose were performed to verify that the animal was producing anti-topo antibody. The IgG fraction was purified on DEAE Affi-Gel Blue according to directions supplied by the manufacturer (Bio-Rad). The IgG was then concentrated by ultrafiltration to 1.7 mg/ml and shown to inhibit relaxation of supercoiled DNA by

purified topo I. The antibody was also shown to: (1) precipitate [³²P]DNA–topo I covalent complexes; (2) precipitate a 105 kDa [³⁵S]methionine-labelled polypeptide from MSB-1 cells; (3) prevent the SDS/K⁺ precipitation of DNA if this IgG (but not control IgG) was preincubated with the 105 kDa topo I. Finally, when this IgG was used to probe Western blots of the 105 kDa topo I, two polypeptides were detected. The predominant one was of 105 kDa, which is the intact form of the enzyme, and a less intense band was observed at 50 kDa; however, we also detected trace amounts of intermediate size polypeptides which are proteolysis products of the intact topo I (Trask & Muller, 1983).

Other methods

Detection of topo I activity by relaxation of plasmid DNA was carried out as described previously (Trask *et al.*, 1984). Preparation of pBR-322 DNA was performed as described (Trask *et al.*, 1984). Western blotting was carried out exactly as described by Towbin *et al.* (1979), with ¹²⁵I-labelled protein A. Polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970).

Results and discussion

Detergent-resistant DNA binding proteins are operationally defined as activities detected by the SDS/K⁺ assay (Trask *et al.*, 1984). The assay was used to follow such activities in nuclear salt extracts, (NH₄)₂SO₄ and Polymix-P fractions. These results can be summarized as follows. The majority of the SDS/K⁺ activities were found in 0.4 M-NaCl extracts of erythrocyte nuclei (0–1 M tested). Higher concentrations of salt liberated significantly more protein, particularly histones, which are inhibitory. When the 0.4 M extract was subjected to (NH₄)₂SO₄ precipitation, we observed that the 0–45% and 45–85% fractions contained substantial activity. The 45–85% fraction contains the 105 kDa topo I (Trask *et al.*, 1984; Trask & Muller, 1983). Furthermore, the 105 kDa topo I appears to be the only SDS/K⁺ activity in the 85% fraction (Trask *et al.*, 1984). The 45% fraction served as a source for the SDS/K⁺ activities. Before (NH₄)₂SO₄ treatment, Polymix-P precipitation was performed to remove nucleic acids. This treatment does not precipitate SDS/K⁺ active proteins.

Chromatographic separation on phosphocellulose is shown in Fig. 1. A major peak of SDS/K⁺ activity was eluted at 0.3 M-(NH₄)₂SO₄ and a second, smaller peak of activity was observed at 0.2 M-(NH₄)₂SO₄. This experiment has been re-

Fig. 1. Phosphocellulose chromatography of SDS/K⁺ activities

(a) Nuclear proteins from erythrocytes were treated with Polymix-P and $(NH_4)_2SO_4$ and applied to phosphocellulose. The column was washed and developed as described in the Materials and methods section. The arrow marks the start of the gradient elution. Fractions (2.5 ml) were collected and the SDS/K⁺ activity, absorbance and conductivity were measured in the indicated fractions. Each SDS/K⁺ assay contained 10 μ l of the fraction. The results are corrected for background precipitation of free DNA. Topo I activity (1 μ l of each indicated fraction) was also assayed by relaxation of supercoiled DNA (b). The positions of relaxed (REL) and supercoiled (SC) DNA are indicated. The first two lanes from the left show plasmid DNA incubated without (–) or with (+) purified topo I. The numbers above the lanes correspond to assays of fractions across the column.

peated four times (different extracts) with essentially the same results. The amount of 0.2M-fraction was somewhat variable each time; however, in all cases it represented only a minor activity peak (or shoulder) compared with the 0.3M-fraction. Both of these fractions contained topo I activity (Fig. 1b), although the 0.3M region had considerably more topoisomerase activity (based upon relaxation of supercoiled DNA). [The 105 kDa form of topo I is eluted from phosphocellulose at about 0.4M- $(NH_4)_2SO_4$ (Trask & Muller, 1983)]. The elution differences between the various forms, although slight, were very reproducible.

The topo I activity associated with the 0.2M and 0.3M SDS/K⁺ peaks was inhibited by pretreatment

with antibody directed against the 105 kDa form of the enzyme (results not shown). These two forms are catalytically active cleavage fragments of the intact (105 kDa) topo I, as reported previously (Liu & Miller, 1981; Trask & Muller, 1983; reviewed by Liu, 1983). When the 0.3M SDS/K⁺ activity was carefully pooled (to avoid contamination of the 0.2M peak) and rechromatographed on the same column, a single peak of activity was eluted again at 0.3M- $(NH_4)_2SO_4$ (results not shown). Thus, with subsequent column steps this activity was not lost or converted to a different form.

The 0.3M pool from phosphocellulose was applied to a hydroxyapatite column. As shown in Fig. 2(a), the SDS/K⁺ activity was eluted at 0.8M-phosphate. In addition, a small amount of activity

Fig. 2. *Hydroxyapatite chromatography*

Fractions 20–27 from the phosphocellulose column were pooled and applied to hydroxyapatite as described in the Materials and methods section. The arrow marks the start of the gradient. SDS/K⁺ assays were performed on 10 μ l of each fraction. The gel in (b) shows the topo I assay data (1 μ l assayed).

was detected in the column flow-through. When the 0.8M-phosphate region was pooled, diluted and rechromatographed over the same column, a flow-through activity was not observed (results not shown). Since the 0.2M pool from the phosphocellulose column (Fig. 1a) does not bind hydroxyapatite, we conclude that the small amount of activity in the flow-through in Fig. 2(a) represents cross-contamination between the 0.2M and 0.3M peaks.

The 0.8M-phosphate pool from hydroxyapatite contains an activity that will relax supercoiled DNA (Fig. 2b). The linking number of a unique DNA topoisomer is altered in steps of 1 by this activity (results not shown); therefore, it is a type I topoisomerase. In addition, the antibody to the 105kDa topo I inhibits both SDS/K⁺ and relaxing activity (results not shown).

The major activity was eluted at 0.8M-phosphate, and contained a prominent polypeptide of 34kDa (Fig. 3a, lane 3). Western blotting was done to determine the relationship of the 34kDa peptide to topo I. Purified IgG directed against the 105kDa form of topo I was used to probe a blot containing the 0.8M-phosphate fraction, purified histone H1, and crude nuclear extracts of erythrocytes or embryonic fibroblasts. The Coomassie Blue-stained gel (Fig. 3a) shows that the 34kDa peptide migrated to the position of histone H1. Identification of the 34kDa peptide as a form of H1 was verified by electrophoresis in acid/urea gels (Bonner *et al.*, 1980). The 0.8M-phosphate fraction contains a single peptide of 50kDa reacting with the antibody (Fig. 3b, lane 3); therefore, the 34kDa peptide is apparently not topo I but is most likely a form of histone H1. Since

Fig. 3. SDS/polyacrylamide-gel electrophoresis and Western blots of active fractions

(a) Marker proteins (lanes 1 and 2), the 0.8M-phosphate pool from hydroxyapatite (lane 3), and purified histone H1 (lane 4) were electrophoresed on a 10% SDS/polyacrylamide gel which was then stained with Coomassie Blue. A second gel was electrophoresed under the same conditions, blotted and probed with antibody directed against the 105 kDa form of topo I; (b) shows the autoradiograph. Lanes 1 and 2 contain a 0.4M-NaCl extract of chicken embryo fibroblasts or erythrocytes, respectively, performed in the presence of proteinase inhibitors (phenylmethanesulphonyl fluoride, α_2 -macroglobulin, aprotinin). Lane 3 contains the 0.8M-phosphate pool from the hydroxyapatite column. Lane 4 contains purified histone H1. The two arrows mark the position of the 105 kDa and the 50 kDa polypeptides. The molecular masses were deduced from markers. An approximate alignment of the stained gel in (a) and the negative in (b) is based upon comparison of the markers in two different gels.

purified H1 did not react with our antibody (lane 4) we can exclude the trivial possibility that we raised antibody to an H1 contaminant in our antigen. Copurification of topoisomerase and H1 has been reported (Pullyblank & Morgan, 1975); however, the data in Fig. 3(b) show that the H1-topoisomerase association is confirmed to a single form of topo I of 50 kDa.

The two predominant forms of topo I (50 and 105 kDa) are present in crude nuclear extracts in erythrocytes and embryonic fibroblasts (Fig. 3b, lanes 1 and 2), and the ratio between the two forms appears similar. (Upon longer exposure of the blot, additional immunoreactive peptides in the range 80–90 kDa were also seen.) The various forms do not appear to be unique to one tissue.

The data in the present paper define conditions in which the SDS/K⁺ assay detects only topoisomerase I in crude nuclear extracts. By altering the reaction conditions, for example by adding cofactors such as ATP or MgCl₂, additional stable

DNA-binding proteins might be detected. We previously reported that purified topoisomerase II/DNA intermediates can be detected by modifying the reaction conditions to include MgCl₂ (Trask *et al.*, 1984).

In conclusion, two SDS/K⁺ activities have been identified which appear to be related to the 105 kDa form of topo I. The most prominent activity (50 kDa) was purified and shown to cofractionate with histone H1. This form was identified previously by cross-linking experiments (Trask & Muller, 1983) and may exist *in vivo* since it is present in crude extracts of nuclei. On the other hand, despite the use of a broad spectrum of proteinase inhibitors, it is conceivable that proteolysis generates the various forms we detect in crude extracts. While it is intriguing that the ratio between the forms appears constant from one tissue to the next, further experiments will be necessary to evaluate the physiological significance of these forms.

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