Alternate domains of neuron DNA topoisomerase I in developing rat brain

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The DNA topoisomerase found in rat brain neurons relaxes supercoiled DNA in the absence of ATP or Mg^{2+}. The estimated content of the active enzyme per nucleus of nerve cell is constant during development from a fetal proliferating neuroblast at the embryonic stage of 18 days to the terminally differentiated neuron (postnatal age of 60 days). The salt stability of DNA topoisomerase association with chromatin varies with the stage of development of nerve cells: at 300 mM NaCl most of the enzyme activity (>90% of the removed activity) elutes from differentiated neuron chromatin, whereas only approx. 25% of the enzyme activity elutes from neuroblast chromatin.

Developing brain *Nerve cell chromatin* *DNA topoisomerase*

1. INTRODUCTION

The topological state of DNA and the enzymes that control this state play a crucial role in determining the function of DNA in cells. A class of enzymes named DNA topoisomerases promote the conversion of one topological isomer into another and are considered to regulate the superhelical conformation of DNA within the cell. The DNA topoisomerases are widely distributed in different cells and mammalian tissues, but not in brain [1,2]. At the same time, neurons offer the unique possibility of studying a single cell type during development from a neuroblast, going from the actively proliferating precursor cell at late fetal stages via a nondividing immature neuron at birth to a terminally and permanently differentiated postmitotic neuron thereafter [3]. This development is accompanied by dramatic changes of the neuron DNA metabolism. In particular, the DNA polymerase α content diminishes correlating temporally with the decline of the in vivo rate of mitotic activity of neuroblasts, and in mature neurons this enzyme is lost completely [4–6]. Because of the absence of replicative synthesis in neuronal DNA coupled with the neuron's lifetime, which is comparable to that of the organism [7,8], brain DNA repair appears to be of prime importance for maintaining the functional integrity of a nerve cell genome [9–12].

Here, we have for the first time found DNA topoisomerase in neurons and observed the alternating salt stability of its association with chromatin during differentiation of nerve cells. Some properties of this enzyme resemble those of type I eukaryotic topoisomerases.

2. MATERIALS AND METHODS

2.1. *Reagents and determinations*

Polyvinylpyrrolidone 40000 (PVP), phenylmethylsulfonyl fluoride (PMSF), poly(dA) and poly(dG) from Sigma were used. Dithiothreitol (DTT) and ethidium bromide were obtained from Calbiochem, crystalline bovine serum albumin fraction V (BSA) from British Drug House, snake venom phosphodiesterase from Boehringer-Mannheim, poly(ethylene glycol) 6000 (PEG) from Loba Chemie, Wien-Fischamend and yeast 8 S RNA from Serva. Supercoiled DNA of PM2 phage was

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isolated and purified according to [13]. Protein concentrations were determined by the technique of [14]. DNA concentration was measured by the diphenylamine reaction as modified in [15].

2.2. *Isolation of neuron nuclei*

Wistar rats of either sex were used. Developmental stages included fetuses of 18 days gestation (−4-day old animals, since the mean duration of gestation in rats is 22 days), as well as animals of postnatal ages 14 and 60 days. Cell nuclei were isolated from forebrain cortex according to a scheme which used intermediate preparation of highly purified neuronal cells, the neuronal perikarya, which upon gentle disruption yield only neuron nuclei [16]. Neuronal perikarya were prepared as described [17] with minor modifications: (1) the cortex preparations were rinsed in an ice-cold standard medium (0.32 M sucrose, 2 mM $MgCl_2$, 1 mM potassium phosphate, pH 6.5) followed by chopping in 7.5% PVP (w/v), 1% BSA (w/v) and 10 mM $CaCl_2$, and (2) the minced brains were filtered through 4 (instead of 3) mesh sizes of nylon cloth (705, 368, 120 and 65 μm). The cell yield decreased in direct proportion with the age of the animals [17]. Therefore, for each isolation different amounts of tissue were taken; 1, 2 and 100 g from fetuses of 18 days, and from 14- and 60-day old animals, respectively, to yield approx. 1.2×10^7 neuron nuclei from animals of each indicated developmental stage. The nuclear preparations obtained varied in protein/DNA weight ratio which increased from 5.3 to 6.9 and 7.1 during animal development; they contained the same amount of DNA, about 7 pg/nucleus. Our neuronal preparations were very similar to those described by others [16,18,19]. The neuronal nuclei were identified as in [16]. The purity of the nuclear preparations as to their neuronal origin was within 94–97%.

2.3. *Preparation of nuclear extract*

The crude nuclear extract was prepared according to [20], supplementing the isolation medium with 0.5 mM PMSF and 0.5 mM DTT. The nuclear fractions, isolated as described above from fetuses of 18 days and from 14- and 60-day old animals, were suspended separately (1×10^6 nuclei of each) in 0.5 ml buffer A (40 mM Tris–HCl (pH 7.5), 1 mM Na_2 EDTA, 0.5 mM PMSF) with 25 mM NaCl. The nuclei were lysed by slow addition, with continuous stirring, of an equal volume of buffer A containing 1 mM DTT, 2 M NaCl and 20% glycerol. The material was sonicated to determine the total amount of DNA topoisomerase activity recovered in the chromatin preparation.

2.4. *Chromatin preparation and salt elution*

The remaining nuclei of each fraction were divided into 5 aliquots (each containing 2×10^6 nuclei). The chromatin was isolated under conditions described in [21]. The resulting crude chromatin preparations were resuspended in 0.5 ml cold buffer A, and an equal volume of the same buffer with 1 mM DTT and 20% glycerol containing either 0.2, 0.6, 1.0, 1.6 or 2.0 M NaCl was added. The DNA in the chromatin extract was precipitated as in [20] by adding 0.5 vol. buffer A containing 0.5 mM DTT, 10% glycerol, 18% PEG and either 0.1, 0.3, 0.5, 0.8 or 1.0 M NaCl, respectively. The DNA with the remaining associated proteins was removed by centrifugation at 17000 × *g* for 20 min at 4°C and the supernatants saved for later assay.

2.5. *Assay of DNA topoisomerase activity*

Topoisomerase relaxation of supercoiled DNA was assayed fluorometrically as described [22] under nonprocessive reaction conditions at 170 mM NaCl. This method allows one to exclude the influence of interfering deoxyribonuclease activities. 2 ml of the sample with variable amounts of enzymic protein in either 0.1, 0.3, 0.5, 0.8 or 1.0 M NaCl were added to 18 ml of the reaction mixture (1 μg of PM2 DNA, 40 mM Tris–HCl (pH 7.5), 1 mM Na_2EDTA and 4 μg BSA) containing 160, 140, 120, 90 or 70 mM NaCl, respectively. The phosphodiesterase solution was supplemented with 1 mM magnesium acetate to protect phosphodiesterase from inactivation by EDTA [23]. One unit of topoisomerase converts 1 μM of PM2 DNA bases to the completely relaxed form in 10 min at 37°C.

3. RESULTS

3.1. *Demonstration of DNA topoisomerase activity in neuronal nuclei of rat brain*

In the beginning, the extract of neuron nuclei

from 14-day old rats, was assayed under standard (for type I eukaryotic DNA topoisomerase) non-processive conditions of supercoiled DNA relaxation. This fraction was characterized by a high DNA relaxing enzymatic activity which was not observed in the absence of monovalent cation. The level of relaxing activity was also assayed in neuroblasts and differentiated neurons. The DNA topoisomerase activity in the nerve cells from rat brain of varying age is shown in table 1. The specific enzyme activity (in units/mg protein) decreased by 30% proportionally with the age of the animal from 18-day fetuses to 60-day old rats. At the same time, the calculations indicate that each nucleus contains an equal amount of the enzyme activity due to the increase of protein/DNA weight ratio in nuclear preparations during animal development, as indicated in section 2.2.

3.2. *Salt elution of the DNA topoisomerase from neuronal chromatin*

DNA topoisomerase is known to be associated with chromatin [1]. We made an attempt to examine the salt dependence of this interaction in nerve cells of 18-day fetuses, 14- and 60-day old rats. For this purpose the enzyme activity in chromatin preparations, obtained as described in section 2.4, was assayed. Separate aliquots of the isolated chromatin were exposed to various concentrations of NaCl for 10 min and the DNA with the remaining associated proteins was removed by PEG precipitation. The enzyme activity eluted from chromatin preparation at 1 M NaCl was defined as 100% and that at other salt concentrations is reported relative to 1 M NaCl eluate.

The salt concentration required to elute the enzyme from chromatin depended strongly on the developmental stage of nerve cells (fig.1). For the differentiated neuron chromatin at least 90% of the activity eluted in 300 mM NaCl, while only 25% of the activity was removed from neuroblast chromatin at this salt concentration. NaCl concentrations of 700 mM or higher were required to remove 90% of the activity from the neuroblast chromatin (fig.1A).

To check for the possible presence of any soluble dissociable activator or inhibitor of DNA topoisomerase with age we examined the salt elution profile of chromatin isolated from a 1 : 1 mixture of neuroblast and differentiated neuron nuclei. We obtained a curve which was intermediate between the two curves shown in fig.1A,C (not shown). These results rule against the possibility and suggest the identity of the conditions for different extract preparations.

Table 1

DNA topoisomerase activity of nerve cell nuclei in developing rat brain

Days of development	Specific activity	
	Units/mg protein	Units × 10^{-7}/ nucleus
−4	33.8 ± 3.9	13.05 ± 1.49
14	27.7 ± 2.6	13.46 ± 1.69
60	25.3 ± 2.1	12.97 ± 1.71

Fractions were prepared from nerve cells of 18-day fetuses (−4-day old rats), 14- and 60-day old rats, as described in section 2.3. PM2 DNA relaxation was assayed as in section 2.5. The values represent the mean of 6 independent determinations

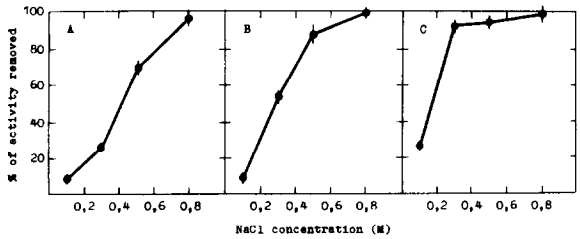

Fig.1. Salt elution profile for DNA topoisomerase from nerve cell chromatin in developing rat brain. The amount of DNA relaxing activity eluted from chromatin extracted from (A) neuroblast (of 18-day fetuses), (B) immature neurons (of 14-day old rats), and (C) terminally differentiated neurons (of 60-day old rats), as a function of NaCl concentration, was measured as described in section 2.5. Each fraction was obtained from aliquots of chromatin preparations extracted by either 0.1, 0.3, 0.5, 0.8 or 1.0 M NaCl as described in section 2.4. The amount of activity found in 1.0 M NaCl chromatin extract was 1 × 10^{-3} units/assay, defined as 100%. The values represent the mean of 4–6 determinations from 2 separate experiments. The bars indicate the range of values of the determined activity.

3.3. *Comparative analysis of the enzymes from nerve cell chromatin of 18-day fetuses, 14- and 60-day old rats*

One possible explanation for the different salt stability for binding of the enzyme to chromatin of neuroblasts, immature and differentiated neurons, might be a difference in structure of the enzyme itself during the process of nerve cell maturation. We examined certain enzymatic properties of the DNA topoisomerase in fractions obtained as described in 2.4 by exposing chromatin to 1 M NaCl. In each fraction the optimal Na^+ or K^+ concentration was 150–200 mM for the relaxing DNA topoisomerase activity. At 50 and 250 mM Na^+ the activities in each fraction were also only 13 and 35% of the maximal activity, respectively. Polynucleotides added to the assay mixture affected DNA topoisomerase activity of each fraction in the same manner (table 2). Poly(dG) and single-stranded phage λ DNA inhibited the enzyme (< 10 and < 18% remaining activity, respectively). However, the inhibition by poly(dG) is reduced by increasing the salt concentration in the assay mixture. Double-stranded phage λ DNA, RNA and poly(dA) did not act on the enzyme.

Table 2

The effect of polynucleotides on relaxation of supercoiled PM2 DNA by the nerve cell DNA topoisomerase in developing rat brain

Added polynucleotides	Relative activity (%) of chromatin fractions		
	I	II	III
Poly(dA)	90	95	93
Poly(dG)	8	7	10
ssDNA (phage λ)	18	15	13
dsDNA (phage λ)	97	93	98
RNA (yeast)	92	91	96

DNA topoisomerase activity was assayed as described in section 2.5 in the presence of 1 μg of the indicated polynucleotides. Fractions I, II and III are 1 M NaCl extracts from nerve cell chromatin of 18-day fetuses, 14- and 60-day old rats, respectively. The activity without additions was approximately 1×10^{-3} units/assay, defined as 100%

4. DISCUSSION

The results obtained indicate the occurrence of DNA topoisomerase in the nerve cell at different stages of maturation. This enzyme, like topoisomerase I from other sources [1,2], relaxes supercoiled DNA in the absence of ATP. In addition, it does not require Mg^{2+} and is active in the presence of 1 mM Na_2 EDTA. The results suggest that the DNA topoisomerase from rat brain neuron chromatin is an enzyme of type I. It should be noted that the most effective inhibition of the enzyme was observed with poly(dG) (90% inhibition) but not with poly(dA) (< 10% inhibition). We have no satisfactory explanation of this fact. However, as reported by authors in [24] calf thymus DNA topoisomerase behaves in the same manner: it can be selectively inhibited by poly(dG) but not by poly(dA), poly(dT) or poly(dC).

A number of biological functions have been proposed for type I DNA topoisomerase. It may participate in DNA replication [25], transcription [26], recombination [27,28], and repair [29]. The postnatal neurons of forebrain are known to have no capacity for replicative nuclear DNA synthesis related to cell division [7,8]. In nuclei of such neurons DNA polymerase β is virtually the exclusive representative of this class of enzymes [4–6]. We have reported a specific exodeoxyribonuclease localized in neuronal cell nuclei from rat brain which appears to be a DNA repair enzyme [12]. We suggest that one of the major functions of DNA topoisomerase in mature neurons is also to participate in the repair of DNA damage, a process essential for postmitotic cells, whose lifetime is comparable to that of the organism. We estimate that each nerve cell nucleus contains approximately 13×10^{-7} units of topoisomerase and this amount is constant during the development of animals from the 18-day fetus to the 60-day old rat. This ratio is similar to that calculated from the data [20,30] for the type I topoisomerase from rat liver (approx. 16×10^{-7} units/nucleus). Thus, the estimated content of topoisomerase per nucleus in postmitotic permanently differentiated neurons is similar to that in proliferating neuroblasts and also in cells of other tissue.

The binding study presented here shows that the salt elution profile of endogenous enzyme from

chromatin of neuroblasts and differentiated neurons appears similar to the salt elution profile of DNA topoisomerase I from chromatin of growing and resting cultivated rodent cells [31]. As in the case of the DNA topoisomerase I activity of mouse 3T3 cell chromatin, more than 70% of the activity remains bound to neuroblast chromatin ('growing' cells) at 300 mM NaCl and 90% of the activity elutes from mature neuron chromatin ('resting' cells) at this salt concentration (fig.1). It appears reasonable to assume that the salt stability is a function of cell developmental stages. Support for this assumption comes from a recent review [2].

The age-related salt stability of the enzyme binding to chromatin of nerve cells cannot be explained by a change of the structure of the enzyme itself during the process of nerve cell maturation since some properties of the enzyme activities in fractions from fetuses of 18 days and from 14- and 60-day old animals are similar, in particular their salt dependence. A possible explanation for this phenomenon is a change in the structure and bulk of proteins mediating the binding of the enzyme during nerve cell development.

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