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DNA POLYMERASE ACTIVITY, PROBABLY DNA POLYMERASE α, REMAINS ASSOCIATED TO MICROTUBULES AFTER SUCCESSIVE POLYMERIZATION CYCLES

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A DNA polymerase activity was found associated to microtubules after three cycles of assembly-dissassembly. The specific activity of such polymerase increases in the assembled microtubule after the first and second cycle and remains constant in the third cycle. The properties of this DNA polymerase resembled the characteristics described for DNA polymerase α.

Microtubules are protein structures found in eukaryotic cells, possibly involved in the intracellular transport of cellular material. It has been reported the association of several molecules and structures to microtubules (1). There are some polypeptides associated in a high proportion to the microtubule (2) and other only detectable by their enzymatic activity (3). It is difficult to distinguish whether the different polypeptides associated to the microtubules are essential components of the biological structure, are associated transiently (i.e. in the intracellular transport), or the association detected is artefactual. However, it is possible to test the relevance of the association of a polypeptide to microtubules, by measuring the relative proportion of the polypeptide to tubulin in successive polymerization cycles.

In a previous work we have found that some of the microtubule associated proteins (MAPs) have a DNA binding capacity which tubulin lacks (4,5) and therefore we have search if there is any activity related with DNA metabolism among the microtubule associated proteins.

In this report I describe that a DNA polymerase activity is associated to microtubules after successive cycles of polymerization.

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MATERIALS AND METHODS

Polyd(A-T)d(A-T) and polyrA$(dT)_{12-18}$ were purchased from P.L. Biochemicals; calf thymus DNA and DNase I were from Worthington, beef liver catalase was obtained from Sigma and [^{3}H]-dTTP was from the Radiochemical Center, Amersham.

Protein fractionation

A porcine brain with a weight of 60 g was homogenized, as described by Shelanski et al (6), in one volume of 0.1 M 2-(N-morpholino)ethane sulfonic acid (MES), pH 6.5, containing 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP (buffer A); the yield was 1,400 mg of soluble protein. Microtubule protein was purified by cycles of polymerization-depolymerization in the presence of glycerol (6) following the centrifugation conditions described by Penningroth and Kirschner (7). After each polymerization step the pellet was resuspended at 4°C in the buffer previously indicated and the DNA polymerase and glyceraldehyde dehydrogenase activities and the amount of protein were measured. Microtubule associated proteins were fractionated from tubulin by chromatography in a DEAE cellulose column, in the conditions of Murphy et al (8). The fractions containing DNA polymerase activity were pooled and salted out with ammonium sulfate. The precipitate of protein was resuspended in buffer A, and chromatographed on a Sephadex G25. The protein fraction excluded from the colum was used for the experiment of Figure 1 as DNA polymerase.

To avoid the disruption of mitochondria and synaptosomes, that may release proteins which can associate to microtubules, the extraction method of Karr et al (9) was also followed.

Assay procedures

DNA polymerase activity was assayed by adding the sample to a buffer containing 35 mM Tris-HCl, 3 mM $MgCl_2$, 0.05 mM EDTA, 3 mM mercaptoethanol, 0.1 mM dATP, dGTP and dCTP, and 0.04 mM [^{3}H]-dTTP, plus 40 µg/ml of calf thymus DNA. The final pH of the mixture was 8.4 and its volume 0.5 ml. The incubation time was 60 min at 37°C and the reaction was stopped by the addition of cold 5% trichloroacetic acid. The specific activity of the [^{3}H]-dTTP was 105 cpm $pmol^{-1}$. One DNA polymerase unit is the amount of enzyme that catalyzes the incorporation of 1 nmol of dTTP into an acid insoluble form in 60 min at 37°C. Glyceraldehyde 3 phosphate dehydrogenase was measured as described by Velick (10), except that cysteine was omitted from the incubation mixture. Protein was determined by the procedure of Lowry et al (11).

RESULTS

DNA polymerase activity was assayed in a microtubule protein solution by testing the incorporation of [^{3}H]-dTTP into acid insoluble material. Table 1 A indicates that such incorporation is due to a DNA polymerase. It requires DNA as template and the four deoxyribonucleoside triphosphates. The incorporated radioactivity is solubilized after incubation with DNase I, and it is not affected by the addition of 0.2 M NaOH.

TABLE 1

TABLE 1A	
Assay mixture	% Incorporation
complete[a]	100
- DNA	2
- dATP and dCTP	7
+ DNase 1[b] (50 µg/ml)	1
+ NaOH 0.2 M[c]	105
TABLE 1B	
+ N-ethylmaleimide[d] (2.5 mM)	19
+ Ethidium bromide (30 µM)	16
+ Phosphonoacetic acid (100 µg/ml)	13
+ ATP (5 mM)	3
TABLE 1C	
Template[e]	
Activated[f]	154
Native DNA	100
Denatured DNA[g]	13
poly d(A-T)d(A-T)	59
polyrA$(dT)_{12-18}$	3

a. Microtubule protein (150 µg) after two polymerization cycles, in the presence of glycerol, was incubated with native calf thymus DNA under the assay conditions described in Methods. a. 100% incorporation was 21 pmol of dTTP incorporated in 60 min. b. Added after 60 min of incubation for an additional 30 min period. c. Added after 60 min, left overnight and folloved by trichloroacetic acid precipitation after neutralization with hydrochloric acid. d. No exogenous mercaptoethanol was added to the assay. e. 40 µg/ml of the different templates resuspended in 15 mM NaCl, 1.5 mM sodium citrate were added. Calf thymus DNA was used as activated, native and denatured DNA 100% incorporation was 25 pmol of dTTP incorporated in 60 min. f. Activated DNA was prepared as described by Aposhian and Kornberg (21). g. Denatured DNA was prepared by heating at 90°C for 10 min followed by rapid cooling and storage at 0°C.

Table 2 shows the incorporation of ^{3}H -dTTP into acid insoluble material when samples, after different cycles of microtubule polymerization, were assayed for a DNA polymerase activity. The results indicate an increase of five times in the specific activity of the enzyme in the assembled microtubule fractions, with a high proportion, (25%) of the total soluble enzymatic activity, associated to the microtubule after three polymerization-depolymerization cycles.

TABLE 2

Experiment	Fraction	DNA polymerase Specific activity u/mg	DNA polymerase Total activity (units)	Glyceraldehyde 3 phosphate dehydrogenase Specific activity u/mg	Glyceraldehyde 3 phosphate dehydrogenase Total activity (units)
1	S_o	0.025	35	6	8.400
	Plg	0.114	18	2	318
	S2g+0.1 mm colchicine		17	-	--
	P2g	0.148	14	0.1	--
2	S_o	0.022 (0.026)	31	41	5.636
	P2	0.110	13	0.1	--
	P3	0.120 (0.136)	8	-	--
DEAE cellulose chromatogr.	not retained	0.83			
	0.3 M KCl	0.00			
	0.8 M KCl	0.00			

Microtubule protein was purified by cycles of assembly-disassembly as indicated in Methods. So, it is the supernatant obtained after the centrifugation at 100,000 x g during 1 h of the homogenate prepared as previously indicated. Plg is the pellet obtained after the first polymerization cycle in the presence of glycerol. S2g and P2g represents the supernatant and the pellet of the second polymerization cycle in the presence of glycerol. P2 and P3 are the pellets of the second and polymerization cycles in the absence of glycerol. The underline numbers indicate the specific activity when the extraction method of Karr et al (9) was followed.

Not retained protein, 0.3 M KCl and 0.8 M KCl indicates the protein fractions obtained when P2 fraction was chromatographed on a DEAE cellulose column indicated by Murphy et al (8).

Table 2 also indicates that the addition of glycerol in the procedure of polymerization, which could modify the proportion of associated protein (12), does not change the amount of DNA polymerase activity found in the microtubule fraction. Moreover, DNA polymerase specific activity also increases when microtubules are obtained following a method which prevents disruption of cell organelles. The addition of 0.1 mM colchicine, known to prevent microtubule polymerization (1), results in a great decrease of the assembled protein after a polymerization cycle. In this case, after centrifugation to fractionate unpolymerized from assembled protein, over 90% of the DNA polymerase activity remains in the supernatant (Table 2).

It has been reported (1,2,7,13,-15) that it is possible to find a large number of enzymatic activities associated to microtubule protein. Also, we have found that several other enzymatic activities are detectable in microtubule protein, but the association of those enzymes with the assembled microtubule seems to be unspecific; i.e. we have found that glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity is present in the assembled microtubule fraction after the first polymerization cycle (Table 2), but on the contrary to DNA polymerase activity, the specific activity associated to microtubules decreases in a successive cycle.

The DNA polymerase activity does not remain associated to tubulin after chromatrography of microtubule protein on DEAE cellulose. When microtubule protein is chromatographed as described by Murphy et al (8), a polypeptide fraction is not retained in the column, while tubulin and some polypeptides associated to microtubules attach to it and can be eluted at 0.8 M and 0.3 M KCl, respectively. Table 2 indicates that DNA polymerase activity is found essentially in the polypeptide fraction which is not retained in DEAE cellulose. This fraction was added in increasing amounts to depolymerized microtubule protein and the mixture was incubated to obtain assembled microtubules. Microtubules were pelleted through a cushion of 50% sucrose

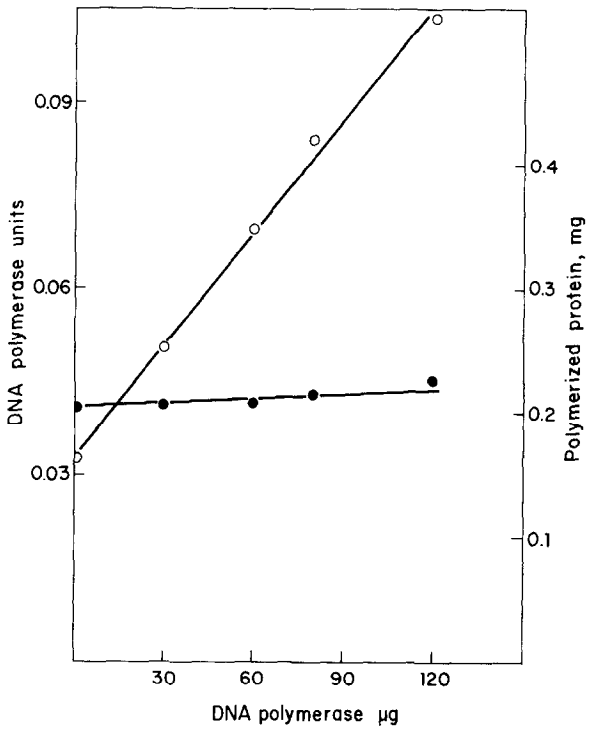

Figure 1. Incorporation of DNA polymerase into pelleted microtubules
Increasing amounts of DNA polymerase (DEAE fraction) were added to 0.25 ml fractions of microtubule protein (2 mg/ml) in buffer A. Microtubules were polymerized by incubating, 30 min, at 30°C, and pelleted through a cushion of 50% sucrose in buffer A at 25°C. DNA polymerase activity (o——o) and protein content of the pellets (●——●) were measured.

in buffer A, and protein content and DNA polymerase activity of the pellets were measured. Figure 1 indicates an increase in the specific activity of DNA polymerase, referred to assembled protein, proportional to the amount of polymerase added; being rather high (from 64% to 72%) the polymerase incorporated into the microtubule, respect to the enzyme added.

Microtubule protein in the cold, in about 0.1 M salt and at neutral pH associates to form complexes of tubulin with MAPs (16). These complexes can be fractionated from the tubulin dimer by filtration on a Sepharose column (17). Figure 2 shows that essentially all the DNA polymerase activity is excluded together with the tubulin-MAP complexes when depolymerized microtubule proteins are chromatographed on a Sepharose 6B column. When 0.1 mM colchicine was added to the microtubule protein before the

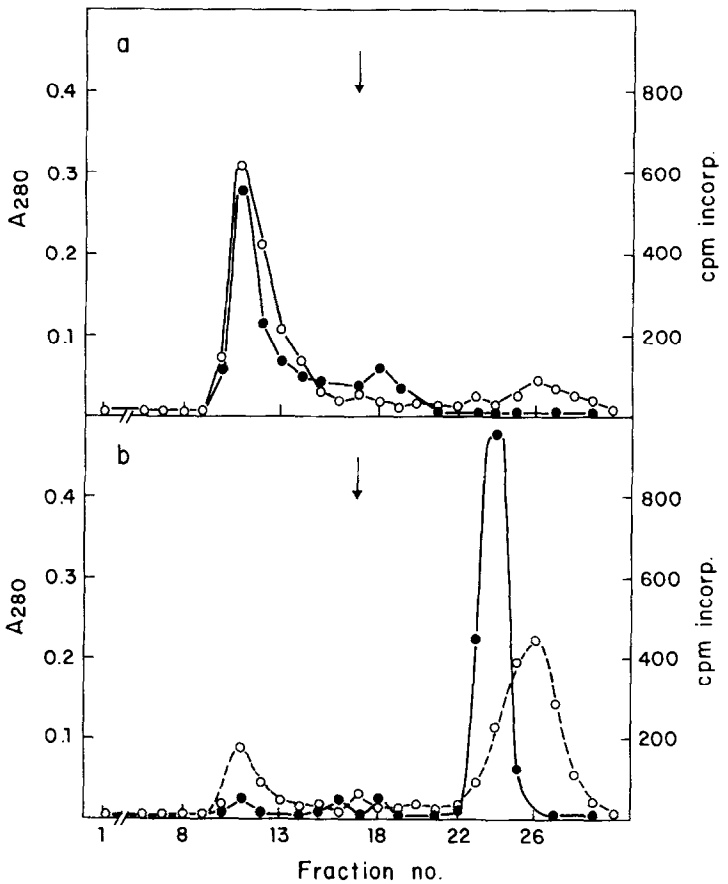

Figure 2. Effect of colchicine in the association of DNA polymerase to microtubule protein. Depolymerized microtubule protein obtained after two polymerization cycles and resuspended (2 mg in 0.5 ml) in 25 mM MES pH 6.5, 1 mM $MgCl_2$, 0.5 mM EDTA and 1 mM 2-mercaptoethanol was chromatographed on a Sepharose 6B column (25 cm x 1 cm). 1.0 ml fractions were collected and the O.D. at 280 nm (o——o) was measured. 0.1 ml of each fraction was used to measure DNA polymerase activity (●——●) in the in the presence of 100 µg of bovine serum albumin. The arrow indicates the elution position of 0.3 mg of beef liver catalase (248,000 M.W.) when it is chromatographed in the same column and followed by measuring the O.D. at 410 nm on each fraction. a) Chromatography of microtubule protein. b) Chromatography of microtubule protein after the addition of 0.1 mM colchicine.

filtration on the column, I found that the proportion of the tubulin dimer peak increases respect to the tubulin-MAPs complex fraction, while the exclusion position of DNA polymerase activity shifts to behave as a globular protein with a M.W. of 130,000. This result suggest that the exclusión of the DNA polymerase activity in the absence of colchicine is not due to the formation of self-aggregation complexes.

Three different classes of DNA polymerases have been described for higher eukaryotic cells of different organisms and tissues, α , β and γ , whose propoerties and localization within the cell varies. In general DNA polymerase α differs from DNA polymerase β in the following characteristics; α polymerase has a molecular weight higher, and β polymerase lower than 100,000 daltons. A fraction of DNA polymerase α is localized in the cytoplasm and is sensitive to different compounds as N-ethylmaleimide, ethidium bromide and phosphonoacetic acid while β polymerase is resistant (i,e. see 18 and 19). The resuts in Table 1B indicate that the polymerase associated to microtubules is sensitive to N-ethylmaleimide, ethidium bromide and phosphonoacetic acid. This result shows that such polymerase does not behave as DNA polymerase β respect to the indicated compounds.

Table 1C indicates the activity of the polymerase when different templates are used. The results in the Table show a higher actitivity with activated and native DNA; such high activity with native DNA could be explained by the presence of an endonuclease in the microtubule preparation.

When polyrA$(dT)_{12-13}$, a very good template for DNA polymerase γ (19), was used, a very low incorporation was found (Table 1C). This result indicates a difference between the DNA polymerase activity associated to microtubules and DNA polymerase γ.

DISCUSSION

The results indicated suggest that DNA polymerase α associates *in vitro* to polymerized microtubules.

A rather high porportion of the total soluble enzyme, about one fourth, was found associated to assembled microtubules after three polymerization cycles. In fact, tubulin, the main microtubule component incorporates about 50% into microtubules after an *in vitro* polymerization cycle (20).

On the other hand, the proportion of G3PDH activity that incorporates into assembled microtubules decreases dramatically with successive polymerization cycles, thus the appearance of G3PDH could be due to a contamination of such activity due to the defficient protein fractionation obtained by the in vitro polymerization method.

These results and the incorporation of exogenous polymerase to microtubules (Fig. 1) suggest and association of a DNA polymerase activity to microtubules. This activity resembles the properties reported for DNA polymerase α (19) and it shows differences with DNA polymerase β and DNA polymerase γ . This late polymerase is abundant in mitochondria (19) and although these organelles may be disrupted in one of the extraction methods used (9), it was not found DNA polymerase γ associated to microtubules. However, DNA polymerase α , that can be found in the cytoplasm as the microtubules, is found associated to these structures.

The association of the DNA polymerase to microtubules could indicate a possible transport of the protein, from the cytoplasm to the nucleus through those structures, based on a higher affinity of the enzyme for polymerized than for unpolymerized microtubule protein.

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