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A DNA POLYMERASE ACTIVITY ASSOCIATED WITH THE SKELETAL FRAMEWORK OF THE PLASMA MEMBRANES OF A RAT HEPATOMA

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SUMMARY: A DNA polymerase activity has been identified in the plasma membranes isolated from a rat hepatoma (the Zajdela Ascitic Hepatoma). The enzyme activity was found specifically associated with the detergent insoluble skeleton of the plasma membranes from which it cannot be dissociated by conventional methods. The properties of the enzyme are indistinguishable from those of DNA polymerase-α

The intracellular location of the eukaryotic DNA polymerase-α has been of considerable recent interest (1-3). Appreciable amounts of the enzyme are invariably found in cytoplasmic fractions in most of the preparative procedures reported so far, although there are some reports available which suggest a nuclear location of the enzyme (3,4). However, it is also known that during mitosis when the cells are transiently anucleate, DNA polymerase is distributed throughout the cytoplasm (3). In addition, there are several reports available which suggest that the enzyme is associated with cytoplasmic membranes such as rough and smooth membranes (5-10). During the course of our studies on plasma membrane proteins of a rat hepatoma, (ZAH cells) we have identified a significant amount of DNA polymerase activity specifically associated with the detergent insoluble skeletal framework of the plasma membranes. We report the results of these findings in this communication.

Abbreviations used: ZAH, Zajdela Ascitic Hepatoma; NP40, nonidet P 40; NEM, N-Ethylamaleimide; PMSF, Phenylmethylsulfonyl fluoride; SDS, Sodium Dodecyl Sulfate.

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EXPERIMENTAL PROCEDURES

Tumour cells. ZAH cells were obtained from Dr F Zajdela, Institut du Radium, Orsay, France and maintained in the laboratory by serial transplantation in Wistar rats. Tumour cells were harvested from the peritoneal cavity between post-transplantation days 5 and 7.

Plasma membrane preparations. The plasma membranes were prepared by two different methods (i) by the biphase method as described by Lesko et.al (11) and (ii) by the sucrose density gradient method as described by Aronson and Touster (12) with minor modifications. In the latter procedure ZAH cells were suspended in 8% sucrose solution and sonicated for 15 sec. at a setting of 80 in a Ralsonics sonicator. The suspension was homogenised in a Potter Elvejehm homogeniser at 1500 rpm by ten strokes. The remaining part of the procedure was the same as that described by Aronson and Touster (12). The membranes obtained by the two methods were suspended in 10 mM Tris, pH 7.6 containing 1 mM PMSF at a protein concentration of 1 mg/ml and frozen at -20° C till further use. Succinic dehydrogenase was assayed as described by Earl and Korner (13) 5'nucleotidase according to the procedure of Gurd and Evans (14) alkaline phosphatase and phosphodiesterase by the procedure of Pekarthy et.al (15), inorganic phosphatase by the method of Shatton et.al (16) and glucose-6-phosphatase by the procedure of Swanson (17).

DNA polymerase assay. The reaction mixture consisted of 50 mM Tris, pH 8.3, 20 mM KCl, 8 mM $MgCl_2$, 25 µg BSA, 1 mM DTT, 25 µg activated calf thymus DNA 0.8 mM each of dATP, dCTP and dGTP; 5 µm [^{3}H] dTTP with a specific activity of 8,000 cpm/pmol,0.05% NP40 and plasma membrane preparations corresponding to 40 µg protein in a total volume of 0.2 ml. [^{3}H] dTMP incorporation in the polymerised product was determined by methods described earlier (18).

Agarose gel chromatography. The tumour cells were swollen in a hypotonic buffer consisting of 1 mM $NaHCO_3$, pH 7.4, 2 mM $CaCl_2$, 1 mM PMSF (buffer A) and lysed gently with 20 strokes using a loose fitting Dounce homogeniser. The homogenate was adjusted to 10 mM Tris, pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 1 mM PMSF (buffer B) and centrifuged successively at 600 x g for 10 min and 10,000 x g for 10 min in order to remove nuclei and mitochondria. The postmitochondrial supernatnat (1 ml containing 3 mg protein) was loaded on a 0.8 x 30 cm Sepharose CL.4B column previously equilibrated with buffer B. The column was eluted with buffer B at a flow rate of 30 ml/hour and 0.5 ml fractions were collected. Aliquots of the fraction (30 µl) were assayed for DNA polymerase activity.

Protein estimation. Protein concentration was estimated by the method of Lowry (19) in the presence of SDS.

RESULTS

Purity of plasma membranes. The purity of plasma membrane preparations was assessed by their marker enzyme profile (Table 1). No detectable cytoplasmic, mitochondrial or endoplasmic reticular contaminations was observed. It was found difficult to assess the purity of ZAH plasma membranes by monitoring the enrichment

TABLE 1

Enzyme activities in ZAH cell plasma membrane preparations

Enzyme	Enzyme activities[a]		
	Crude homogenate	Preparation 1[b]	Preparation 2[c]
5'nucleotidase	ND[d]	0.095	0.055
Alkaline phosphatase	ND	0.056	0.075
Phosphodiesterase-I	0.018	0.215	0.178
Glucose-6-phosphatase	0.011	ND	ND
Succinic dehydragenase	0.112	ND	ND
Pyrophosphatase	0.400	ND	0.001

[a]Enzyme activity is expressed in terms of units per milligram protein. One unit equals 1 μmol of substrate reacted per minute. Assay procedures used for the different enzymes are referred to in the text.

[b]Plasma membranes prepared by the sucrose density gradient method of Lesko et.al (11)

[c]Plasma membranes prepared by the sucrose density gradient method of Aronson and Touster (12)

[d]Not detectable

of 5' nucleotidase commonly used as a marker for the plasma membranes, because of the low level of the enzyme present in the ZAH cells. However, an estimate of the purity of the plasma membranes can be obtained from the fact that succinic dehydrogenase and glucose-6-phosphatase activities were undetectable in the preparations. Furthermore, ZAH plasma membranes show a protein profile on SDS-polyacrylamide gel very similar to that of rat liver plasma membranes for which data on these marker enzymes are available (not shown).

Association of DNA polymerase-α -like activity with plasma membranes. The purified plasma membranes were assayed for DNA polymerase activity as described in the Experimental Procedures. Use of low concentration (0.05%) of non-ionic detergent was

TABLE 2

Substrate utilisation by membrane bound DNA polymerase

Template	Incorporation of [^{3}H] dTMP pmol/hr
Activated calf thymus DNA	1,250
Activated calf thymus DNA+NEM(1 mM)	150
Poly dA oligo dT	938
Poly rA oligo dT	6
Poly rC oligo dG	8

Membrane samples (50 µg protein) were assayed with different templates in a total volume of 0.2 ml . Synthetic templates were used at 20 µg/ml while activated calf thymus DNA was present at 25 µg/ml in the reaction mixture. Activities were expressed as pmoles of [^{3}H] dTMP incorporated per mg protein.

essential to detect the enzyme activity, while higher detergent concentrations (> 0.4%) were found inhibitory for the enzyme reaction. The polymerase activity could not be solubilised from the membranes by different treatments such as sonication solubilization with high salt(1M NaCl) or detergents (1% NP40) or by any combination of these procedures. The solubilisation of the membrane preparation with NP40 removed about 80% of the membrane proteins, but all the polymerase activity could be recovered in the insoluble pellet. The protein profile obtained when this pellet was analysed by SDS-polyacrylamide gel electrophoresis was distinctly different from that exhibited by the 1% NP40 supernatant (not shown).

The results of template specificity and NEM-sensitivity experiments are shown in Table 2. These results suggest that the membrane bound enzyme has properties very similar to those of DNA polymerase-α.

In order to check whether the observed association of the polymerase activity with the membranes was a result of any artifactual association during membrane isolation, we have carried out independent checks using gel permeation chromatography (20).

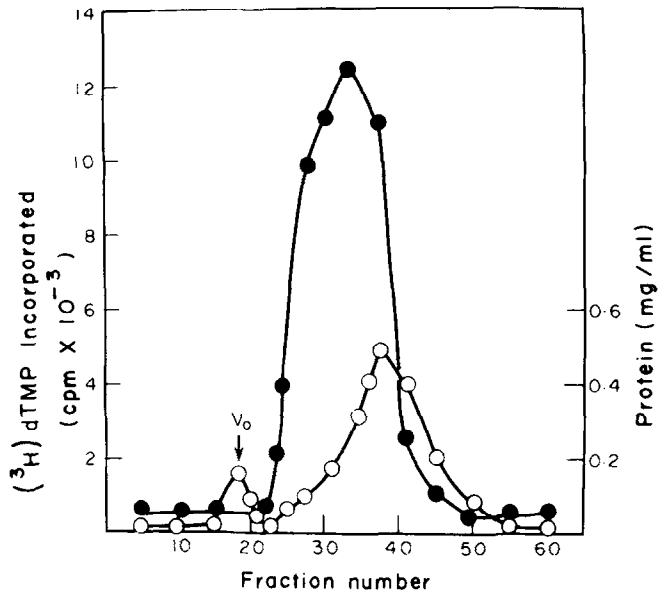

FIGURE 1 Sepharose CL.4B column profile of postmitochondrial supernatant prepared from ZAH cells. ●-● enzyme activity. O-O protein profile. Arrow indicates position of void volume (Vo).

When the postmitochondrial supernatant of ZAH cells was fractionated on 4% agarose (Sepharose CL.4B) soluble DNA polymerase activity eluted immediately after the void volume (Fig.1). The fractions containing any material eluting at the void volume (fractions 16 to 22, Fig.1) showed no DNA polymerase activity. However, when these fractions were pooled and centrifuged at 105,000 x g for 90 min enzyme activity could be readily detected in the pellet on treatment with NP40 (0.05%). This activity corresponded to about 5-8% of the total DNA polymerase activity present in the cells (Table 3).

When the active fractions eluting after the void volume (fractions 25-45, Fig.1) were similarly pooled and centrifuged, there was no observable pellet and all the polymerase activity was recovered in the supernatant. When this was concentrated and rechromatographed on a second agarose column, all the activity eluted after the void volume. These results suggest that the enzyme activity detected in the void volume was not

TABLE 3

Distribution of DNA polymerase activity on elution from Sepharose CL.4B column

Sample	Total Protein (mg)	Total activity (pmol[^{3}H] dTMP incorporated)	% activity recovered
Load (post mitochondrial supernatant)	3.0	7,000	100
Post void volume fractions[a]	2.52	6,287	89.8
Void volume fractions[b]	0.256	560	8.0

[a]Fractions 25-45 of Fig.1

[b]Fractions 16-22 of Fig.1

due to aggregation or artifactual association of the soluble enzyme with membranes.

DISCUSSION

We have demonstrated the presence of DNA polymerase activity indistinguishable from DNA polymerase-α, in the detergent insoluble residue of plasma membranes isolated from ZAH cells. The specific association of the enzyme with the detergent insoluble residue of the plasma membranes is demonstrated by several observations: (i) the enzyme activity could not be detected in the membranes without the use of a nonionic detergent (ii) the enzyme could not be solubilised from the membranes by any of the conventional methods or a combination of these methods; (iii) fractionation on agarose column clearly demonstrated the presence of the activity in the membraneous fraction present at the void volume of the column, quite separate from the soluble cytoplasmic enzyme.

The existence of a detergent insoluble matrix in plasma membranes which could serve as a membrane skeletal network has been demonstrated for lymphoid cells (21). We have also observed the presence of similar structures in plasma membranes from rat

hepatocytes (unpublished observations). The detergent insoluble membrane skeleton forms a part of an elaborate network of filaments as 'cytoskeletal framework' extending from the plasma membranes to the nucleus (22). Several biological activities such as mRNA translation (22) and phosphorylation by the src gene product (23) are known to be associated with this structure. In light of these observations, it becomes important to understand the significance of the association of DNA polymerase with the skeletal framework of the plasma membranes.

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