DNA-DEPENDENT ATPases IN CONCANAVALIN A STIMULATED LYMPHOCYTES

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1. Introduction

Since DNA replication depends on ATP [1], one would expect to find in replicating cells enzymes which utilize ATP in the presence of DNA. Several DNA-dependent ATPases have been found in bacterial cells [2–6] but only one corresponding activity has been detected in mammalian cells [7]. The relation of this latter activity to the replication process is unknown. This communication reports the isolation and characterization of three different DNA-dependent ATPases from proliferating lymphocytes. The increase in the activity of two DNA-dependent ATPases and the appearance of a third one during the transition from resting to proliferating lymphocytes indicates a possible role for these enzymes in the replication process. A preliminary abstract of this work has already been presented [8].

2. Materials and methods

2.1. *Preparation, cultivation and stimulation of lymphocytes*

Preparation, cultivation and stimulation of lymphocytes from bovine retropharyngeal lymphnodes were carried out as reported elsewhere [9]. Single-stranded DNA cellulose, containing 1.5 mg calf thymus DNA/g cellulose [10] and [γ-^{32}P]ATP [11] were prepared as described.

2.2. *Preparation of DNA-dependent ATPases from lymphocytes*

Standard buffer contained 50 mM Tris–HCl, pH 8.0, 2 mM EDTA, 20% (v/v) glycerol and 10 mM 2-mercaptoethanol. If not stated otherwise all operations were carried out at 4°C. 5 g packed resting or proliferating lymphocytes (isolated 50 h after stimulation by Concanavalin A) were suspended in final vol. 20 ml in standard buffer without glycerol and treated five times by a loose fitting Dounce homogenizer. 5 ml 2 M ammonium sulfate solution was added. After sonication for 10 s (Braun sonic 300S, 25% max. speed) the viscous suspensions were mixed by magnetic stirring for 30 min. After centrifugations for 60 min at 15 000 × *g*, the supernatants were diluted with standard buffer and glycerol to final concentrations of 20% glycerol and 0.2 M ammonium sulfate. Nucleic acids were removed by passing the fractions through DEAE-cellulose columns (50 ml) previously equilibrated with standard buffer containing 0.2 M ammonium sulfate. The flow-through fractions were dialysed against 500 ml standard buffer containing 25 mM NaCl for 18 h and then applied to single-stranded DNA cellulose columns (20 ml) equilibrated with the same buffer. Proteins were eluted with 1 M NaCl in standard buffer, concentrated by ammonium sulfate precipitation (67% saturation) and further purified on linear sucrose gradients as described [12]. In other experiments, the protein fractions were dialysed against standard buffer containing 50 mM NaCl and applied to DEAE-cellulose columns. The DNA-dependent ATPases were eluted with 250 mM NaCl in standard buffer.

2.3. *Enzyme assays*

ATPase assay: 2–10 μl fractions were incubated for 30 min at 37°C in 100 μl reaction mixtures containing 50 mM Tris–HCl, pH 8.0, 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 50 μg bovine serum albumine, 2 μg denatured calf thymus DNA and 100 μM [γ-^{32}P] ATP (10–100 cpm/pmol). Reactions were stopped by

the addition of 500 μl a suspension of 50 ml powdered charcoal (Aktivkohle, Merck) in 500 ml distilled water. After vigorous shaking, charcoal was removed by centrifugation. The [^{32}P]phosphate (inorganic phosphate and pyrophosphate) not adsorbed to charcoal in 100 μl of the supernatants were determined.

Assays of DNA polymerase [13], endo- and exonuclease [12] and DNA ligase activities [14] were done as described. Assays of ATP dependent unwinding activities were carried out according to Abdel-Monem et al. [5] using DNA–RNA hybrid duplexes which had been prepared as previously described [12].

3. Results

DNA-dependent ATPases were isolated both from resting lymphocytes and from proliferating lymphocytes, harvested at the time of optimal DNA synthesis (50 h after stimulation by Concanavalin A), using a simple assay which measures the release of ^{32}P-radioactivity from ATP in the presence of DNA. In fig.1, the distribution of activities from both resting and proliferating cells on linear sucrose gradients is compared. The data clearly demonstrate a large increase in DNA-dependent ATPase activity in activated, replicating relative to dormant lymphocytes. Two fast-sedimenting activities from proliferating lymphocytes are very unstable and could not be presented for further characterization.

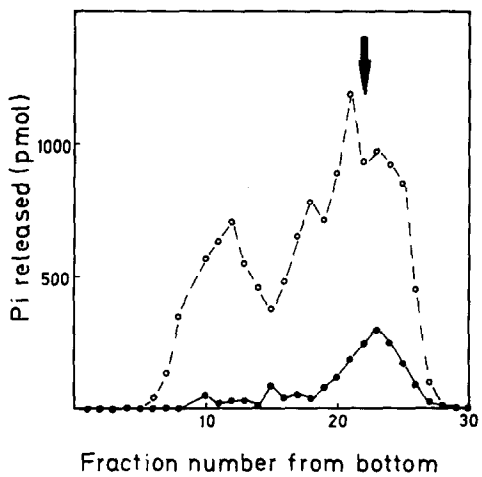

Fig.1. Comparison of DNA-dependent ATPase activities from resting and proliferating lymphocytes. Fractions after DNA cellulose chromatography from resting (•–•) and proliferating lymphocytes (○– –○) were centrifuged through linear sucrose gradients [12] and assayed for ATPase and DNA polymerase activities as described in Materials and methods. The arrow indicates the sedimentation position of DNA polymerase α.

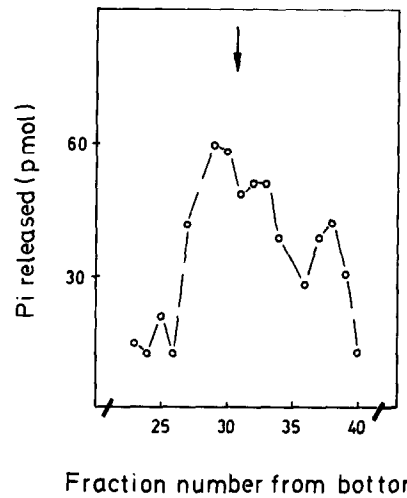

Fig.2. Sedimentation behaviour of DNA-dependent ATPases from proliferating lymphocytes. The DEAE-cellulose salt eluate fraction from proliferating lymphocytes was centrifuged after its concentration by ammonium sulfate precipitation (67% saturation) through linear sucrose gradients [12] and fractions were assayed on ATPase (○–○) and DNA polymerase activities as described in Materials and methods. The arrow indicates the sedimentation position of DNA polymerase α.

Figure 2 shows the distribution of DNA-dependent ATPases from proliferating lymphocytes on linear sucrose gradients after further purification by DEAE-cellulose chromatography. Three different activities can be seen, one of which sediments slightly faster than the DNA polymerase from the same cell preparation. On the basis of their sedimentation behaviour these activities were named ATPase I (8 S), ATPase II (6 S) and ATPase III (4 S) and were further characterized.

Competition experiments showed that all three activities cleave ATP and dATP preferentially over all other nucleoside triphosphates and deoxytriphosphates. Analysis of the reaction products by poly-(ethylene–imine)-cellulose chromatography revealed that all three activities catalyze the conversion of

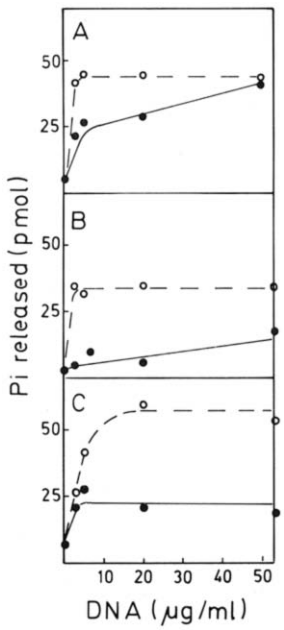

Fig.3. Dependence of ATPase. The activities of ATPase I (A), ATPase II (B) and ATPase III (C) were measured in a standard assay depending on increasing concentrations of double-stranded (●–●) or single-stranded DNA (○– –○) as indicated.

ATP and dATP to ADP and dADP, respectively, and inorganic phosphate. All activities depend on Mg^{2+} ions and on the presence of DNA. Figure 3 illustrates the preference of ATPase I for single-stranded over double-stranded DNA. ATPases II and III seem to be rather specifically stimulated by single-stranded DNA and resemble in that respect an ATPase isolated from mouse myeloma [7]. The ATPases also differ from each other in K_m for ATP and optima for ionic strength, as summarized in table 1.

ATPases II and III can be isolated from resting lymphocytes, and their activities increase by a factor of about 5 during the transition from resting to proliferating lymphocytes (see table 1). In contrast ATPase I is recovered only in proliferating lymphocytes; resting cells apparently do not contain this enzyme. Similary in G_0-arrested mouse ascites cells ATPases II and III, but not ATPase I were recovered.

4. Discussion

In this paper, I report the existence of three DNA-dependent ATPases in proliferating lymphocytes. These enzymes were enriched by binding to single-stranded DNA cellulose. Similar enzymes with a lower affinity to DNA may therefore be removed during this purification step. Indeed a DNA-dependent ATPase was found in the flow-through of the DNA cellulose columns, which after concentration by phosphocellulose chromatography and centrifugation through linear sucrose gradients could be characterized as a polynucleotide ligase [14]. This ATPase activity depends on the presence of nicked, double-stranded DNA.

The three DNA-dependent ATPase activities described here differ in several biochemical properties. However, most interesting is the fact that these activities increase

Table 1
Properties of DNA-dependent ATPases

	Sedimentation coefficients	K_m (mM ATP)	Optimal NaCl concentrations (mM)	Activity increase: stimulated lymphocytes / resting lymphocytes
ATPase I	8 S	0.025	50	> 20
ATPase II	6 S	0.025	10	5
ATPase III	4 S	0.150	50	5

Sedimentation coefficients were calculated from the migration rate of the activities on linear sucrose gradients taking DNA polymerase α (7 S) as a sedimentation marker. K_m Values for ATP were obtained by extrapolations from double reciprocal plots of activities versus ATP concentrations; optimal ionic strengths were measured using the standard ATPase assays (see Materials and methods) in the presence of varying amounts of NaCl. The activities of DNA-dependent ATPases from resting and proliferating lymphocytes were calculated after the purification steps described in Materials and methods and are based on comparable protein concentrations of the fractions before the DNA cellulose purification step.

many-fold when resting lymphocytes are activated to proliferate by Concanavalin A; in fact, ATPase I activity was not detected at all in resting cells. This may indicate a possible participation of these enzymes in the replication process. I have investigated whether these DNA-dependent ATPases have additional enzymatic functions such as nuclease- or DNA-unwinding activity, but all attempts so far have failed.

Acknowledgements

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