DNA-Dependent Adenosinetriphosphatase A: Immunoaffinity Purification and Characterization of Immunological Reagents†

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ABSTRACT: We describe an immunoaffinity purification of DNA-dependent ATPase A from fetal calf thymus. The rapid purification increases the yield of enzymatically active enzyme approximately 4-fold, with up to a 7-fold increase in specific activity, and significantly improves the yield of a higher molecular weight species of ATPase A. In the presence of a denatured calf thymus DNA effector, the immunoaffinity-purified enzyme has a specific activity that is more than 10-fold higher than reported for any other eukaryotic DNA-dependent ATPase and 100-fold higher than most others. The improvement in yield has allowed several polypeptides to be identified using monoclonal antibodies, and these polypeptides are demonstrated to be structurally related by partial peptide mapping with *N*-chlorosuccinimide. The preferred DNA effector for ATP hydrolysis continues to be a DNA primer–template junction with an adjacent stretch of single-stranded DNA. We have used the immunoaffinity-purified enzyme to develop additional stable murine hybridoma monoclones, resulting in a bank of antibodies that recognize a number of different epitopes. All of the monoclonal antibodies react with both calf thymus DNA-dependent ATPase A and bacteriophage T4 gene 44 protein, a DNA-dependent ATPase essential for DNA replication in the bacteriophage T4 system. These monoclonal antibodies should facilitate the development of our understanding with respect to the role and regulation of DNA-dependent ATPases in eukaryotic DNA replication.

The role of DNA-dependent ATPases has been established in both replication and recombination of prokaryotic DNA by a number of genetic and enzymatic approaches (Kornberg & Baker, 1992). While similar enzymes have been identified in eukaryotic organisms (Mesner et al., 1991, and references therein), significant progress in understanding these enzymes has been impeded by the lack of available genetics. A comparable problem occurs for many studies of other eukaryotic proteins, but alternatives other than genetic approaches have been successfully employed. For instance, monoclonal antibodies have played a critical role in the purification, molecular cloning, and understanding of eukaryotic DNA replication-associated proteins such as DNA polymerase α (Tanaka et al., 1982; Wahl et al., 1984, 1988; Wong et al., 1988). Furthermore, studies of prokaryotic DNA-*in*dependent ATPases have shown significant progress toward understanding the regulation of ATP hydrolysis and protein–protein interactions through the use of monoclonal antibodies (Aggeler et al., 1990).

Recognizing the critical role that DNA-dependent ATP hydrolysis may play in DNA replication, we have previously reported the development of three monoclonal antibodies (Mesner et al., 1991). We report here the use of these MAbs[1] to develop an immunoaffinity purification protocol for DNA-dependent ATPase A. Using the most efficient DNA effector (poly(dA-dT)) for DNA-dependent ATPase A, we find a specific activity that is as much as 25-fold higher than any previously reported for eukaryotic DNA-dependent ATPases (Boxer & Korn, 1980; Thomas et al., 1988) and that closely approximates that of some highly purified prokaryotic DNA-dependent ATPases (Lohman et al., 1989; Wood & Matson, 1987). Thus, this preparation of ATPase A appears to yield protein of sufficient purity for further analysis.

We have developed a battery of monoclonal antibodies against calf thymus ATPase A and have screened these for cross-reactivity with antigens from other systems. These antibodies appear to recognize a number of epitopes which are also available in the bacteriophage T4 gene 44 protein, an integral part of the DNA replication machinery in bacteriophage T4. Consequently, these results reinforce our conclusion that DNA-dependent ATPase A is the eukaryotic analogue of the gene 44 protein.

MATERIALS AND METHODS

Materials. All the reagents used were analytical grade. Protease inhibitors and myosin were purchased from Sigma Chemical Co. *Escherichia coli* recA protein and bacteriophage T4 gene 32 protein were purchased from US Biochemicals. *E. coli* DNA polymerase I Klenow fragment was prepared according to Joyce and Grindley (1983). Bacteriophage T4 gene 44/62 and 45 proteins were a gift from Dr. Peter von Hippel (Jarvis et al., 1989a). *E. coli* DNA helicases I, II, and IV were kindly provided by Dr. Steven W. Matson. *E. coli* DNA replication proteins τ and γ were generously donated by Dr. Charles S. McHenry. Anti-DNA-dependent ATPase A monoclonal antibodies (MAbs) were developed by the University of Virginia Lymphocyte Culture Center according to the protocol of Chapman et al. (1984). Primary and booster injections of mice for development of the monoclonal antibodies used 0.2–1.0 unit of fraction IV immunoaffinity purified ATPase A (Table I). Monoclonal antibodies used for analytical purposes were isolated from ascites fluid (Reik et al., 1987) and purified to homogeneity

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; MAb, monoclonal antibody; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylene diaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; NCS, *N*-chlorosuccinimide.

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Table I: Immunoaffinity Purification of DNA-Dependent ATPase A

fraction	protein (mg)	activity (units)	sp act. (units/mg)	yield (%)	purification (x-fold)
I. crude	36000[a]	b	b	(100)	(1)
II. DEAE-cellulose	4465	b	b	(100)	(13)
III. ssDNA–cellulose	59	14	0.24	100	600
IV. 6E12-Affi-Gel Hz	0.014	2.4	171	17	427 500

[a] The purification shown was begun with 600 g of calf thymus glands. [b] Fraction III is the first fraction with which it is possible to identify DNA-dependent ATPase activity. The values in parentheses have been estimated by assuming 100% recovery of the activity during the initial steps of this purification.

on protein G–Sepharose CL4B (Pharmacia-LKB) according to the manufacturer's protocol.

Chromatography Resins. Denatured DNA–cellulose was prepared from calf thymus DNA and Whatman CF-11 cellulose as previously described (Alberts & Herrick, 1971). DEAE-cellulose (DE-52) was purchased from Whatman. Affi-Gel Hz was purchased from Bio-Rad, and monoclonal antibodies were coupled to the resin according to the manufacturer's protocol.

Electrophoretic Procedures. Gel electrophoresis in the presence of SDS and subsequent silver staining of proteins was performed according to published procedures (Dreyfuss et al., 1984; Morrissey, 1981). High and low molecular weight markers were purchased from Bio-Rad. Biotrace NT (Gelman, 0.45-μm pore size) nitrocellulose membranes were used in dot-blot and electrophoretic-transfer procedures. Western blotting was performed by a published procedure (Towbin et al., 1979), and blots were probed with 5 μg/mL of MAb, unless otherwise indicated. Antigen–antibody complexes were detected with horseradish peroxidase conjugated F_c-specific goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and the colorimetric substrate diaminobenzidine (tetrahydrochloride salt). Rainbow high and low molecular weight markers (Amersham) were used on gels designated for western blotting.

N-Chlorosuccinimide Digests. Following purification by the protocol described in Table I and gel electrophoresis, the 105-kDa polypeptide of ATPase A was excised from a 10% SDS–Tricine gel (Schagger & von Jagow, 1987). The 83- and 68-kDa polypeptides were excised from gels following purification by the published protocol (Mesner et al., 1991). The gel slices containing the 105-, 83-, or 68-kDa polypeptide were subjected to partial cleavage with NCS (Lischwe & Ochs, 1982) and subsequently electrophoresed on a 10% SDS–Tricine–polyacrylamide gel (Schagger & von Jagow, 1987). DNA-dependent ATPases B, C, and D isolated by the method of Hockensmith et al. (1986) were treated in solution with NCS according to the method of Lischwe and Ochs (1982), dialyzed, and denatured prior to electrophoresis on a 10% SDS–Tricine gel. High and low molecular weight Rainbow (Amersham) markers were used for SDS–Tricine gels.

Immunoprecipitations. The antibody-Affi-Gel Hz bead complex was incubated with a fraction containing DNA-dependent ATPase activity for 2 h at 4 °C, precipitated by centrifugation, rinsed with buffer, and subsequently assayed for ATPase activity. ATPase assays have been previously described (Hockensmith et al., 1986). One unit of ATPase activity is defined as the amount of enzyme that can hydrolyze 1 μmol of ATP/min at 37 °C.

Affinity Constants. Antibody–antigen binding constants were estimated using the modified ELISA assay of Friguet et al. (1985) with the correction delineated by Stevens (1987) for the use of whole antibodies. Immunoaffinity-purified ATPase A (fraction IV, Table I) was used for all determinations.

Other Methods. Protein concentration was determined using a published procedure with bovine serum albumin as a standard (Bradford, 1976). DNA helicase, DNA polymerase, and DNA polynucleotide kinase assays were performed as previously described (Hockensmith et al., 1986).

Immunoaffinity Purification of DNA-Dependent ATPase. An immunoaffinity purification protocol was developed (Table I) using MAb 6E12-Affi-Gel Hz column chromatography. All steps were performed at 0–4 °C. Buffers were prepared using distilled, deionized water.

(A) Preparation of Thymus Gland Extract. Frozen fetal calf thymus glands were broken into pieces weighing ~15 g. The pieces were blended in a Waring blender (full speed for 2 min), using 170-g portions with 500 mL of AB buffer (20 mM Tris-HCl, pH 7.5, 5% (w/v) glycerol, 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, and 5 mM β-mercaptoethanol) containing 0.7 μg/mL leupeptin and 0.7 μg/mL aprotinin. Immediately after homogenization, PMSF and pepstatin A were added to a final concentration of 0.5 mM and 0.7 μg/mL, respectively. The homogenate resulting from 600 g of thymus tissue was sedimented for 30 min at 9000 rpm (13500*g*) in a Sorvall GS-3 rotor. The resulting supernatant (~1850 ml) was decanted and passed through four layers of cheesecloth (fraction I).

(B) DEAE-Cellulose Chromatography. Fraction I was divided into six aliquots, and each was incubated with 150 ml of DEAE-cellulose equilibrated in AB buffer. The DEAE-cellulose suspension was placed on a rotator for 1 h at 4 °C to provide gentle mixing. The cellulose was subsequently sedimented for 10 min at 2500 rpm (1056*g*) in a Sorvall GS-3 rotor. The supernatant was decanted and discarded. The 900 mL of DEAE-cellulose was resuspended in ABP buffer (AB buffer containing 0.5 mM PMSF) and subsequently packed into a column (8.3 × 17 cm). The column was then washed with 2 L of ABP buffer, followed by elution with 2 L of 200 mM NaCl in ABP buffer (fraction II). We have previously reported that ATPase A does not bind to DEAE-cellulose (Hockensmith et al., 1986), and our immunoaffinity-purified enzyme retains this characteristic. Thus we attribute the binding of fraction I ATPase to either protein–nucleic acid interactions or protein–protein interactions. Elution of the DEAE-cellulose column with salt concentrations greater than 200 mM NaCl results in a fraction which will not flow properly over subsequent columns, an observation consistent with elution of nucleic acids from the DEAE-cellulose.

(C) Denatured DNA–Cellulose Chromatography. Fraction II was adjusted to a conductivity of less than 9.5 mS, using ABP buffer without NaCl. The resulting solution was loaded at 600 mL/h onto a denatured calf thymus DNA–cellulose column (6.2 × 22 cm) previously equilibrated with ABP buffer. The column was then washed with 1.5 L of AP buffer (ABP buffer without β-mercaptoethanol). The column was subsequently eluted with AG buffer (20 mM Tris-HCl, pH 7.5, 20% (w/v) glycerol, 5 mM EDTA, 5 mM EGTA, and 0.5 mM PMSF) containing 1 M NaCl (fraction III).

(*D*) *Immunoaffinity Chromatography*. A saturated solution of ammonium sulfate was added to fraction III until the resulting solution was at 70% of saturation (PMSF was the only protease inhibitor present in the saturated $(NH_4)_2SO_4$ solution used for fractionations). After being mixed, this suspension can be stored overnight at 4 °C prior to centrifugation in a Sorvall GS-3 rotor for 10 min at 9000 rpm (13500*g*). The pellet was resuspended and diluted in AG buffer containing 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin, and 0.7 μg/mL pepstatin A but without NaCl. Dilution was continued until a conductivity of approximately 15 mS was attained. The resulting solution was loaded at 20 mL/h onto a guard column of Affi-Gel Hz (1.25 × 10 cm) connected to a column of monoclonal antibody 6E12·Affi-Gel Hz beads (1 × 8 cm) containing 0.65 mg of MAb 6E12/mL of Affi-Gel Hz. Each column was previously equilibrated in AG buffer. The Affi-Gel Hz guard column removes at least one protein from the preparation that would otherwise bind to the 6E12·Affi-Gel Hz beads but does not bind the DNA-dependent ATPase, thereby allowing the enzyme to flow onto the 6E12·Affi-Gel Hz and bind. The adjoined columns were washed with 1 column volume of 550 mM NaCl in AG buffer. After the two columns were detached, the 6E12·Affi-Gel Hz column was washed extensively with 550 mM NaCl in AG buffer and subsequently eluted with buffer G (20 mM Tris-HCl, pH 7.5, 20% (w/v) glycerol, 1 mM EDTA, 2 M NaCl, 1.4 M $MgCl_2$, and 0.5 μg/mL each of leupeptin and aprotinin). The resulting fraction was dialyzed three times for 1 h each time in 1 L of AG buffer. Following dialysis, the enzyme was concentrated in a Centricon-30 concentrator (Amicon). Repeated cycles of dilution and concentration were performed on fraction IV until the conductivity of the filtrate was equivalent to the conductivity of 50 mM NaCl in AG buffer (3.0 mS at 4 °C). Attempts to concentrate the enzyme prior to dialysis have always led to loss of enzymatic activity.

RESULTS

Purification of calf thymus DNA-dependent ATPase A by conventional column chromatography has previously yielded two polypeptides (68 and 83 kDa) that are immunologically related and which appear to be eukaryotic analogues of the essential replication-associated gene 44 protein in bacteriophage T4 (Hockensmith et al., 1986; Mesner et al., 1991). The multiple species of DNA-dependent ATPase A along with the relatively low yields (ca. 4%) obtained from our prior purification schemes have led us to develop an immunoaffinity purification protocol for DNA-dependent ATPase A.

The heterogeneity of the antigens in DNA–cellulose fractions fostered our desire to alter our purification protocol. We prepared each of our three MAbs, 2D1, 4B10, and 6E12, as affinity matrices by chemically coupling each to Affi-Gel Hz beads. We found that each of the antibody–bead complexes can be used to precipitate greater than 80% of the DNA-dependent ATPase activity from a purified preparation of 83-kDa ATPase A. The ATPase–MAb·Affi-Gel Hz complex was stable to washes with salt concentrations up to 550 mM NaCl in AG buffer, while the control Affi-Gel Hz beads (no antibody) could be washed free of ATPase activity with 50 mM NaCl in AG buffer (data not shown). Each of the antibody–enzyme complexes was competent for DNA-dependent ATP hydrolysis, indicating that none of these antibodies were occluding the ATP- or DNA-binding domains of the enzyme. Since the 6E12 antibody does not bind to the smallest form of ATPase A (68 kDa) and its binding to the denatured form of the enzyme is weak (Mesner et al., 1991;

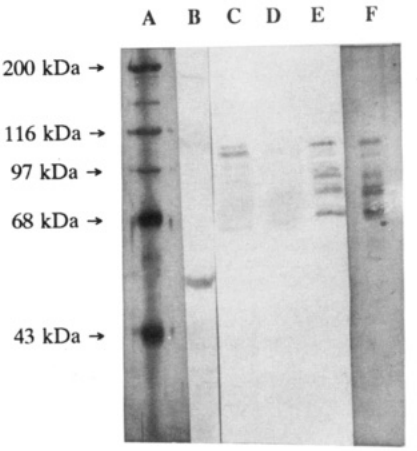

FIGURE 1: Silver-stained gel (A, F) and western blot analysis (B–E) of fractions from the purification protocol in Table I. SDS–polyacrylamide gels (8%) were loaded with 0.25 μg (each) of molecular weight standards (A), 75 μg of fraction II from DEAE-cellulose (B), 15 μg of fraction III from denatured DNA–cellulose (C), 15 μg of the 6E12·Affi-Gel Hz column flow-through (D), 20 ng of fraction IV (E), or 340 ng of fraction IV (F). Immobilized protein was probed with MAb 2A3.

Table III), we chose the 6E12·Aff-Gel Hz bead as the chromatographic matrix to use in the immunoaffinity purification of DNA-dependent ATPase A. Attempts to use the 4B10 or 2D1·Affi-Gel Hz beads for immunoaffinity purification have found that the antigens can only be eluted under denaturing conditions (low pH or SDS).

Immunoaffinity chromatography of fraction III from Mesner et al. (1991) on 6E12·Aff-Gel Hz beads resulted in a fraction that contained a number of polypeptides with apparent molecular weights between 75 and 105 kDa (data not shown). Each of the high molecular weight polypeptides reacted with each of the three previously reported MAbs (2D1, 4B10, 6E12) (Mesner et al., 1991). We have repeated our purification numerous times with a host of protease inhibitors, including PMSF, leupeptin, pepstatin A, *p*-aminobenzamidine, ε-aminocaproic acid, chymostatin, aprotinin, and 1,10-phenanthroline, as well as EDTA and EGTA, which are normally present in all of our buffers. We find that none of these proteolytic inhibitors alter the antigenic composition of our immunoaffinity-purified ATPase A, nor do they increase the yield of active ATPase A.

Myriad attempts to reduce the purification of DNA-dependent ATPase A to a method yielding a single high molecular weight protein have led to the purification protocol shown in Table I. We have used a batch-adsorption to DEAE-cellulose procedure similar to that of Lee et al. (1984) to generate fraction II containing DNA-dependent ATPase A. Western blots of fraction II reveal a band of 105 kDa with no detectable 68- or 83-kDa product (Figure 1, lane B). Thus chromatography of fraction II on single-stranded, denatured, calf thymus DNA cellulose also results in a fraction (III) that is highly enriched for the 105-kDa polypeptide, although smaller products are detectable on a western blot. A comparison of equivalent fractions from our previous protocol (Mesner et al., 1991) and the DEAE-cellulose batch adsorption reveals the distinct improvement in yield of higher molecular weight species afforded by the latter method (data not shown). Chromatography on the 6E12·Affi-Gel Hz matrix yields a high specific activity DNA-dependent ATPase A which is enriched for the 105-kDa polypeptide species. The immunoaffinity chromatography step yields approximately a 700-fold increase in purity and reduces the time required for

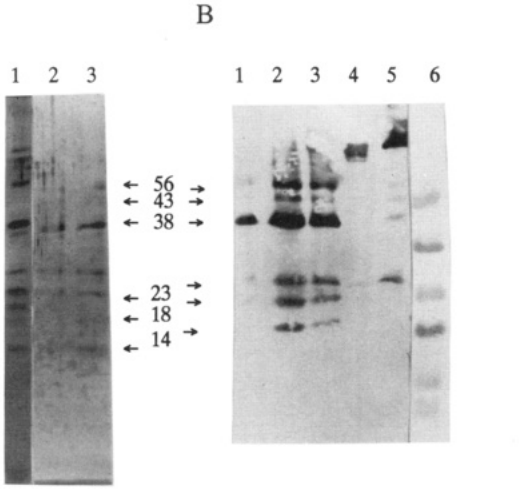

FIGURE 2: *N*-Chlorosuccinimide partial digests of ATPase A. (A) Silver-stained SDS–Tricine–polyacrylamide gels showing NCS digests of the 68-kDa (lane 1, 0.016 unit), 83-kDa (lane 2, 0.158 unit), and 105-kDa polypeptides (lane 3, 0.123 unit). (B) Western blot showing NCS digests of the 68-, 83-, and 105-kDa polypeptides (lanes 1–3, respectively) and the undigested 68- and 83-kDa polypeptides (lanes 4 and 5, respectively). Lane 6 shows Rainbow molecular weight markers (Amersham) of 46, 30, 21.5, 14.3, 6.5, and 3.4 kDa. Immobilized protein was probed with MAb 4B10.

purification from ~10 days to ~36 h. The entire protocol increases yields to approximately 17% (Table I) and yields an overall 4- to 7-fold improvement in specific activity when compared to our previous protocol (Mesner et al., 1991).

A possible scenario for the multiple antigens in the immunoaffinity-purified ATPase A is that they represent proteolytic products derived from a high molecular weight parent polypeptide. The 68-, 83-, and 105-kDa polypeptides were prepared and subjected to denaturing gel electrophoresis. The resulting bands were excised, and the proteins were subjected to *N*-chlorosuccinimide digestion followed by electrophoresis. As shown in Figure 2, each of the polypeptides shares common fragments, thereby providing further evidence that each was derived from the same parent.

The multiple species of ATPase A not only are structurally related but also are functionally similar. An extensive discussion of the DNA effector preference for ATP hydrolysis by ATPase A was presented by Hockensmith et al. (1986). From the considerable characterization reported on the 68-kDa ATPase A (Hockensmith et al., 1986), we have chosen to compare a sampling of the effectors which are descriptive of the enzyme's properties. Table II demonstrates that the effector preferences of the 68-kDa ATPase A and the immunoaffinity-purified ATPase A fractions are virtually identical, supporting the conclusion that the 68-kDa enzyme and the fraction IV immunoaffinity-purified enzyme have identical effector preferences: a primer–template junction with a segment of adjacent single-stranded DNA. Furthermore, no polymerase, helicase, or polynucleotide kinase activity was detected in either of the DNA-dependent ATPase A preparations.

Unlike the structural and functional similarities that exist for the different forms of ATPase A, the enzymatic stability varies significantly. The 68- and 83-kDa DNA-dependent ATPase A fractions are very stable when stored at 4 °C (half-life = 3 years), but fraction IV ATPase A has a half-life of 10 days at 4 °C. Consequently, fraction IV DNA-dependent ATPase A should be stored at –80 °C in small aliquots. Fraction IV enzyme undergoes a 3% loss of activity per freeze–thaw cycle, but otherwise can be stored indefinitely at –80 °C.

There have been a number of reports of chromatographically distinct DNA-dependent ATPases from eukaryotic cells (Assairi & Johnston, 1979; Hockensmith et al., 1986; Tawaragi et al., 1984). Efforts to chromatographically resolve the individual polypeptide species of fraction IV ATPase A have failed, but earlier we had demonstrated that a number of DNA-dependent ATPases could be resolved by DNA–cellulose chromatography, yielding DNA-dependent ATPases A, B, C, and D (Hockensmith et al., 1986). Figure 3 demonstrates that these ATPases are all recognized by our monoclonal antibodies against ATPase A and thus are antigenically related to ATPase A. Confirmation of the relatedness of ATPases B, C, and D to ATPase A was achieved through NCS digests which yielded the same polypeptides as shown for ATPase A in Figure 2 (data not shown).

We have used immunoaffinity-purified ATPase A to develop additional MAbs (Chapman et al., 1984). Ten MAb-secreting hybridomas were isolated (Table III). Each of the new antibodies reacted strongly with immunoaffinity-purified ATPase A in an ELISA assay (>2 A_{414nm} units), and each also reacted with the 68-kDa ATPase A.

Immunoaffinity ATPase A was assayed for ATP hydrolysis in the presence of each of the MAbs, and the results are shown in Table III. Similar to the findings of Aggeler et al. (1990) for *E. coli* F_1 ATPase, none of our antibodies yield greater than 30% inhibition of ATPase activity, and many stimulate ATP hydrolysis up to 50%. We have now coupled MAbs 10E8, 6E2, 3B2, 1H10, and 5G4 to Affi-Gel Hz beads and find that each can be used to quantitatively precipitate DNA-dependent ATPase A activity. As before, these ATPase A–bead complexes were stable to washes of up to 550 mM NaCl in AG buffer and were also stable to washes with 20 mM Tris-HCl, pH 7.5, and deionized water (data not shown). Furthermore, incubation of the ATPase A–bead complexes with 2 mM ATP, 200 μM denatured calf thymus DNA, or the complete ATP hydrolysis reaction mixture did not cause any detectable release of the enzyme from the bead complexes. Since ATPase activity becomes associated with the beads, we conclude that the epitopes for the binding of each antibody are accessible to the MAbs in solution and also that these antibodies are specifically anti-ATPase A IgGs.

All of the MAbs except 1H10 test positive as IgG_1 isotypes. The MAb 1H10 has an IgG_{2a} isotype. MAbs 6E2 and 4H11 appear to have mixed isotypes since they not only are IgG_1 positive but also appear to be IgA and IgG_{2a} positive, respectively (Mesner, 1992). A summary of the binding affinity for the MAbs is presented in Table III. The individual binding affinities of the different MAbs vary over a 15-fold range that is very similar to that found for DNA polymerase α (Tanaka et al., 1982). Similarly to the case of DNA polymerase α antibodies (Tanaka et al., 1982), we have been unable to correlate binding affinity with either an inhibitory or a stimulatory effect on the ATP hydrolysis activity of ATPase A. As we have previously shown in a qualitative manner (Mesner et al., 1991), MAb 6E12 has a relatively low binding affinity, which accounts for its utility in the purification of ATPase A. Attempts to establish binding affinities for MAbs 6E2, 4H11, and 2D1 using the modified ELISA have been unsuccessful. The relative response of these three antibodies to ATPase A on dot blots would seem to indicate that their affinity falls within the range of affinities shown in Table III.

Table II: Strand Effector Characteristics of 68-kDa ATPase A and Immunoaffinity ATPase A

nucleic acid[a]	nmol of P_i formed (% of control)		nmol of P_i formed, 100 mM added NaCl (% of control)	
	ATPase A[b]	immuno-ATPase A[c]	ATPase A	immuno-ATPase A
none	<1.0	<1.0	<1.0	<1.0
denatured calf thymus DNA	100	100	81	91
poly(dA-dT)·poly(dA-dT)	151	191	76	143
$p(dT)_{16}$	nd[d]	<1.0	1.5	<1.0
poly(dA)	5.2	2.5	4.1	<1.0
$p(dT)_{16}$·poly(dA), 1:1	68	78	37	46
$p(dT)_{16}$·poly(dA), 1:5	66	60	48	54
$p(dT)_{16}$·poly(dA), 5:1	56	72	5.7	10

[a] Each nucleic acid was present at a concentration of 200 μM (nucleotide) in the standard ATPase reaction mixture. [b] Hockensmith et al. (1986) *Biochemistry 25*, 7812–7821. DNA-dependent ATPase A hydrolyzed 131 nmol of ATP per hour at 37 °C using denatured calf thymus DNA as the effector. [c] Values derived from the average of three determinations (SD ≤ ±5%). Immuno-ATPase A hydrolyzed 104 nmol of ATP per hour at 37 °C using denatured calf thymus DNA as the effector. [d] Not determined.

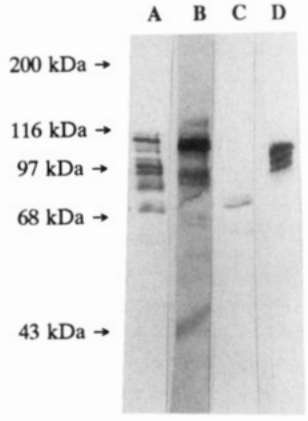

FIGURE 3: Western blot of ATPases A, B, C, and D (Hockensmith et al., 1986). Lane A, 0.02 μg of immunopurified ATPase A (fraction IV). Lane B, 1.03 mg of ATPase B. Lane C, 0.53 μg of ATPase C. Lane D, 0.17 μg of ATPase D. Immobilized protein was probed with a mixture of the 13 antibodies (2.5 μg/mL each) reported in Table III.

Table III: Monoclonal Antibody–ATPase A Dissociation Constants

monoclone	% activity[a]	binding affinity, K_d
2A3	101	$(5.50 \pm 0.33) \times 10^{-10}$
5G4	138	$(6.68 \pm 0.43) \times 10^{-10}$
1H10	125	$(8.94 \pm 1.28) \times 10^{-10}$
5A12	97	$(9.78 \pm 0.80) \times 10^{-10}$
3B2	122	$(1.01 \pm 0.08) \times 10^{-9}$
7B1	154	$(1.08 \pm 0.09) \times 10^{-9}$
10H7	71	$(1.25 \pm 0.16) \times 10^{-9}$
10E8	126	$(1.25 \pm 0.16) \times 10^{-9}$
4B10	114	$(1.49 \pm 0.20) \times 10^{-9}$
6E12	123	$(7.95 \pm 2.69) \times 10^{-9}$
2D1	88	nd[b]
6E2	127	nd
4H11	118	nd

[a] One microgram of each MAb was separately incubated with 0.0013 unit of DNA-dependent ATPase A for 0.5 h at 0 °C in a volume of 10 μL. Ninety microliters of the standard reaction mixture was added, and the samples were incubated at 37 °C for 20, 40, and 60 min. ATP hydrolysis rates were determined from a linear least-squares determination of the indicated time points; correlation coefficients were ≥0.990. ATP hydrolysis in the absence of MAb was designated as 100%. [b] Not determined.

Unlike other DNA-dependent ATPases, ATPase A and the bacteriophage T4 gene 44 protein show a unique preference for primer–template junctions as effectors of ATP hydrolysis (Hockensmith et al., 1986; Jarvis et al., 1989b). We have tested all of the MAbs against the gene 44 protein. We were surprised to find that every MAb reacted with the gene 44 protein and that all MAbs reacted within the range of gene 44 protein concentrations that we had previously established (Mesner et al., 1991). The cross-reactivity of the ATPase A MAbs with the gene 44 protein does not appear to be a phenomenon of general cross-reactivity with most or all ATPases or adenine nucleotide binding proteins. We obtained a number of proteins and subjected them to dot-blot analysis using the MAbs. The MAb 4B10 routinely yields a positive dot-blot analysis detecting 1–3 fmol of ATPase A. Detection of gene 44 protein requires 0.3–1 pmol, or approximately 300 times as much epitope as for ATPase A. A number of *E. coli* proteins, including helicase I, helicase II, τ, and γ, require in excess of 10 pmol for detection using MAb 4B10 and at best yield a response that is marginally positive. The DNA-*in*dependent ATPase myosin and non-ATPase DNA binding proteins such as bacteriophage T4 gene 32 protein and *E. coli* DNA polymerase I Klenow fragment yield similar marginally positive results when the amount of protein is in the 10–100-pmol range. We have found only one protein that is recognized in a fashion similar to gene 44 protein: the DNA-dependent ATPase *E. coli* helicase IV. Much like gene 44 protein, helicase IV can also be detected in the range of 0.3–1 pmol of protein. Since we have been unable to detect any helicase activity with ATPase A, and since the effector preferences of ATPase A and helicase IV are vastly different (Hockensmith et al., 1986; Wood & Matson, 1987), we do not consider ATPase A to be an analogue of helicase IV. While we have not tested all of the MAbs for cross-reactivity with these various antigens, we generally find that the binding affinities for the various antigens follow the same pattern as discussed for MAb 4B10. The single exception is MAb 3B2, which appears to cross react with any protein containing both a polynucleotide binding site and nucleotide binding site (e.g., DNA-dependent ATPases, polymerase, ligases, etc.) and which is the subject of additional investigations.[2]

DISCUSSION

Eukaryotic DNA replication proteins have been reported previously to be sensitive to proteolysis with the generation of multiple proteolytic species (Lee et al., 1984; Sauer & Lehman, 1982; Siegal et al., 1992). Subsequent development of immunoaffinity purification protocols has significantly aided the purification of the unproteolyzed proteins (Wahl et al., 1984). On the basis of these previous reports and the lack of genetics in vertebrate systems, we have developed a bank of MAbs with the intent of establishing the size of the parent ATPase A polypeptide, along with an immunoaffinity purification protocol. Initial attempts at identifying antigens in excess of 83 kDa by probing western blots of crude DNA-

[2] L. D. Mesner, P. A. Truman, and J. W. Hockensmith, manuscript in preparation.

dependent ATPase A fractions led to the identification of a number of 4B10 antigens in the first DNA–cellulose fraction. In addition to 68- and 83-kDa proteins (Hockensmith et al., 1986; Mesner et al., 1991), the western blots showed two predominant antigens with calculated molecular masses of 85 and 105 kDa. After establishing that MAbs 2D1, 4B10, and 6E12 could be used to immunoprecipitate DNA-dependent ATPase activity from the first DNA–cellulose fraction, we designed an immunoaffinity purification protocol using MAb 6E12. The initial fractionation of a calf thymus extract frequently results in substantial proteolysis, and consequently, one of the key features of this purification protocol is the use of DEAE-cellulose chromatography, which yields a fraction containing the 105-kDa antigen.

The DEAE-cellulose chromatography must be performed at the beginning of the purification procedure because the purified DNA-dependent ATPase A will not bind to DEAE-cellulose (Hockensmith et al., 1986). Previously we have used DEAE-cellulose chromatography to remove residual DNA fragments after elution of ATPase A from a DNA–cellulose column (Hockensmith et al., 1986). Such a step is no longer necessary since the immunoaffinity protocol outlined in Table I yields an ATPase A fraction that is completely DNA-dependent. We presume that any residual DNA is stripped from the ATPase during the high-salt washes of the enzyme bound to the 6E12-Affi-Gel HZ column.

The 6E12 immunoaffinity-purified ATPase A fraction is a high specific activity enzyme (171 units/mg) that represents a significant improvement in purity and a dramatic decrease in the time required for enzyme preparation. Furthermore, we have continued to report our specific activity using denatured calf thymus DNA as an effector. However, the best effector for DNA-dependent ATPase A is poly(dA-dT), which results in an increase in the specific activity to 267 units/mg and rivals the specific activity of both DNA-*in*dependent ATPases (F_1 ATPase; Aggeler et al., 1990) and prokaryotic DNA-dependent ATPases (*E. coli* Rep helicase; Lohman et al., 1989). Our protocol results in approximately a 4-fold increase in the yield of total DNA-dependent ATPase activity compared to fractions from our previously reported purification method and contains the same predominant antigen (105 kDa) that appeared in the DEAE-cellulose fraction. We have used the immunoaffinity-purified ATPase A to develop additional antibodies and find that all of the MAbs recognize the same proteins in the DNA–cellulose fraction. Furthermore, all of the MAbs except 6E12 recognize the 68-kDa ATPase A.

The ATP hydrolysis activity of fraction IV ATPase A displays DNA-dependence and DNA-effector properties that are characteristic of the 68-kDa DNA-dependent ATPase A. The antigenic properties, enzymatic properties, and peptide maps of fraction IV ATPase A lead us to conclude that all of the polypeptides in this fraction are DNA-dependent ATPase A or products thereof.

Our attempts to preferentially isolate the hypothetical parent DNA-dependent ATPase A by the addition of a number of protease inhibitors in the purification protocol or by a number of different protocols have proven unsuccessful. In particular, we had hoped to resolve the different species of ATPase by chromatography on DNA–cellulose (Hockensmith et al., 1986; Mesner et al., 1991), but were surprised to find that the affinity of immunoaffinity-purified ATPase A for DNA–cellulose was apparently higher than for less pure fractions of the enzyme.

Aggeler et al. (1990) have provided data for the *E. coli* F_1 ATPase and demonstrated that some antibodies specifically stimulate ATP hydrolysis possibly by competition for a binding site normally occupied by the ϵ subunit, an inhibitory protein. The high specific activity of our immunoaffinity-purified ATPase (Table I), the increased affinity of this immunoaffinity-purified ATPase for DNA, and the increased resistance of the ATPase activity to added salt (Table II) are all consistent with the removal of an inhibitory subunit during our immunoaffinity chromatography. The results we have obtained thus far suggest that the intact DNA-dependent ATPase A is a 105-kDa protein. We have not eliminated the possibility that the parent polypeptide of ATPase A could have a higher molecular weight because we have been unable to detect antigens in crude extracts from calf thymus tissue.

On the basis of these observations, we propose that DNA-dependent ATPase A is at least a 105-kDa protein that is readily degraded to a stable "core" protein (68 kDa) and the 105-kDa polypeptide possesses all of the ATP-hydrolytic properties characteristic of DNA-dependent ATPase A. The 68-kDa ATPase A is the smallest protein that we have isolated, and the additional amino acid sequence which the higher molecular weight polypeptides possess does not affect the effector for DNA-dependent ATP hydrolysis. We speculate that the additional protein sequence (and the secondary and tertiary structure which it confers) may be involved in associations with other proteins. Some DNA-dependent ATPases which play a role in DNA replication exert their influence on this process by apparently assembling other proteins into a multisubunit protein complex competent for processive DNA replication (Kornberg & Baker, 1992). A specific example is the DNA-dependent ATPase known as the gene 44 protein from bacteriophage T4. The gene 44 protein forms a complex with the gene 62 protein and binds to DNA primer–template junctions in the presence of the gene 45 protein. It has been postulated that the energy released by the hydrolysis of the β–γ phosphodiester bond of ATP may provide the energy to assemble the remaining components of the replication complex (Jarvis et al., 1989b). Thus the MAbs that stimulate DNA-dependent ATPase A activity may be mimicking a yet unidentified ATPase A-associated protein (as opposed to removing an inhibitory subunit; see above).

We have viewed proteolysis as detrimental but acknowledge that the multiple antigens that we detect and purify (i.e., the 68-, 83-, and 105-kDa proteins) could be physiologically relevant proteins that share enzymatic and antigenic properties but differ in some undetermined way. We have explored a wide range of methods for the purification of this enzyme as a single, high molecular weight species and have exhausted many of the traditional chromatographic techniques used to study proteins. Nevertheless, the immunoaffinity purification protocol yields immunologically pure antigens that we have shown to yield common peptides upon partial digestion, thus yielding a rich source of oligopeptides for amino acid sequencing.

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