Diadenosine Tetraphosphate Binding Protein from Human HeLa Cells: Purification and Characterization†

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ABSTRACT: The ubiquitous dinucleotide P^1,P^4-di(adenosine-5′) tetraphosphate (Ap_4A) has been proposed to be involved in DNA replication and cell proliferation, DNA repair, platelet aggregation, and vascular tonus. A protein binding specifically to Ap_4A is associated with a multiprotein form of DNA polymerase α (pol α_2) in HeLa cells. The Ap_4A binding protein from HeLa cells has been purified to homogeneity starting from pol α_2 complex. The Ap_4A binding protein is hydrophobic and is resolved from the pol α_2 complex by hydrophobic interaction chromatography on butyl-Sepharose and subsequently purified to homogeneity by chromatography on Mono-Q and Superose-12 FPLC columns. The Ap_4A binding activity elutes as a single symmetrical peak upon gel filtration with a molecular mass of 200 kDa. Upon polyacrylamide gel electrophoresis under nondenaturing conditions, the purified protein migrates as a single protein of 200 kDa. Upon electrophoresis under denaturing conditions, the binding activity is resolved into two polypeptides of 45 and 22 kDa, designated as A_1 and A_2, respectively. A_1 and A_2 can be cross-linked using the homobifunctional cross-linking agent disuccinimidyl suberate. The cross-linked protein migrates as a single protein of 210 kDa on polyacrylamide gels under denaturing conditions, suggesting that these two polypeptides are subunits of a single protein. The purified protein binds Ap_4A efficiently, and by Scatchard analysis, we have determined a dissociation constant of 0.25 μM, indicating high affinity of Ap_4A binding protein to its ligand. ATP is not required for the binding activity. The nonionic detergent Triton X-100 is necessary for stabilizing the purified protein. Amino acid composition analysis indicates that A_1 and A_2 are distinct.

P^1,P^4-Di(adenosine-5′) tetraphosphate (Ap_4A)[1] is a member of a special group of dinucleotide polyphosphates that are ubiquitous in prokaryotic and eukaryotic cells. Ap_4A is synthesized as a product of the back-reaction of the amino acid activation step by aminoacyl-tRNA synthetase [reviewed in Baril et al. (1985)]. As a pleiotropic cell growth effector, it has been proposed to be involved in DNA replication, DNA repair, cell proliferation, platelet aggregation, and vascular tonus (Andersson, 1989). Intracellular levels of Ap_4A increase with cellular proliferative activity (Rapaport & Zamecnik, 1976), and the increase in Ap_4A is correlated with the onset of DNA synthesis (Morioka & Shimada, 1985; Weinman-Dorsch et al., 1984). Ap_4A induces DNA replication in permeabilized, quiescent baby hamster kidney cells (Grummt, 1978), and when microinjected to *Xenopus laevis* oocytes (Zourgui et al., 1984). Poly(ADP-ribosylated) Ap_4A inhibits Simian virus 40 DNA replication in vitro (Baker et al., 1987). A potential role of Ap_4A in DNA replication is inferred by the identification of a Ap_4A binding protein associated with pol α from calf thymus (Grummt et al., 1979; Rapaport & Feldman, 1984), HeLa cells (Baril et al., 1983), and *Xenopus laevis* (Zourgui et al., 1988). Pol α plays a major role in lagging-strand DNA replication (Thommes & Hubscher, 1990) and is associated with accessory proteins such as DNA primase and primer recognition proteins (Vishwanatha et al., 1986). To understand the physiological role of the dinucleotide Ap_4A in the process of DNA replication and cell proliferation, we have undertaken the purification and characterization of the Ap_4A binding protein. Previously, Ap_4A binding protein has been purified from HeLa cells (Baril et al., 1983) and calf thymus (Rapaport & Feldman, 1984). However, the previously published protocols were not efficient in our hands to obtain homogeneous preparations. The protocol described in this report results in reproducible good yields of pure protein that has enabled us in the characterization of this protein.

Experimental Procedures

Cells and Cell Growth. HeLa S3 cells were maintained in suspension cultures in Joklik's minimal essential medium supplemented with 5% defined iron-supplemented calf serum (Hyclone) at 37 °C. Cells were harvested at a density of (0.5–1.0) × 10^6 cells/mL, while they were in the log phase of growth. Cells were monitored for mycoplasma contamination by using a Mycotrim TC detection system (Hana Biologics, Inc.). Cell pellets were rinsed twice with Hank's balanced salt solution and stored at −80 °C until further use.

Homogenization of HeLa Cells and Preparation of Crude Extract. Fifty grams of frozen cells was thawed and resuspended in 150 mL of homogenization buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF) and incubated on ice for 10 min. Cells were homogenized with a pestle B and a Dounce homogenizer followed by centrifugation of the homogenate at 2000 rpm for 10 min (Sorvall SS-34 rotor). The supernatant cytoplasmic fraction was further centrifuged at 45 000 rpm at 4 °C for 1 h (Beckman 60 Ti rotor), and the supernatant at this step was saved as cytosol. The nuclear pellet from the first centrifugation was resuspended in 150 mL of nuclear extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, and 0.15 M KCl) and rotated on a Nutator for 1

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[1] Abbreviations: Ap_4A, P^1,P^4-di(adenosine-5′) tetraphosphate; pol α, DNA polymerase α.

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h at 4 °C and then centrifuged at 45 000 rpm for 1 h at 4 °C (Beckman 60 Ti rotor). The supernatant was saved as nuclear extract. The cytosol and nuclear extract were combined as "crude extract". An appropriate amount of KCl was added to the crude extract to give a final concentration of 0.15 M before the first chromatography column.

Buffers. The following buffers were used in purification of the binding protein: TDE, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1 mM EDTA; TDA, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.8 M ammonium sulfate; TDEG, TDE buffer containing 10% (v/v) glycerol.

Protein Purification by Column Chromatography. (A) DEAE-cellulose Column. HeLa crude extract, prepared as described above, was first subjected to chromatography on a 25-mL DEAE-cellulose (DE-52 grade, Whatman) anion-exchange column, equilibrated with TDEG buffer containing 0.15 M KCl. The DE-52 column was then washed with 300 mL of TDEG buffer containing 0.15 M KCl. The unbound fraction, containing most of the pol α activity and all of the Ap_4A binding activity, was concentrated to 100 mL by using an Omegacell (Pharmacia) concentrator (fraction I).

(B) DNA–Cellulose Columns. Fraction I was applied to a 10-mL native DNA–cellulose column (Sigma) connected in series to a 10-mL single-stranded DNA–cellulose column (Sigma). The coupled columns were preequilibrated with TDEG buffer containing 0.15 M KCl. After fraction I was loaded, the columns were washed with 150 mL of TDEG buffer containing 0.15 M KCl. Under these conditions, all of the pol α activity and the Ap_4A binding activity remain unbound and flow through the coupled columns. The flow-through fraction was concentrated to 150 mL using an Omegacell concentrator and dialyzed against TDEG buffer (fraction II).

(C) DEAE Bio-Gel Column. Fraction II was applied to a 50-mL DEAE Bio-Gel column (Bio-Rad) equilibrated with TDEG buffer. The column was then successively washed with 250 mL each of TDEG, TDEG containing 0.15 M KCl, and TDEG containing 0.3 M KCl. The TDEG/0.15 M KCl fraction (fraction III) contained all of the Ap_4A binding activity and represents the multiprotein pol α_2 complex described before (Vishwanatha et al., 1986).

(D) Butyl-Sepharose Column. Fraction III was dialyzed against TDA buffer and applied to a 15-mL butyl-Sepharose column (Pharmacia) equilibrated with TDA buffer. The column was washed with 150 mL each of TDA, TDE, and TDE containing 0.5% Triton X-100. The TDE/0.5% Triton X-100 eluate (fraction IV), containing most of the Ap_4A binding activity, was dialyzed against TDEG buffer and concentrated to 150 mL.

(E) Mono-Q FPLC Column. Fraction IV was applied to a Mono-Q FPLC column (Pharmacia) equilibrated with TDEG buffer. The sample was applied by multiple injections through a 20-mL superloop. The flow-through fraction containing unbound proteins was collected. Proteins bound to the column were eluted with a 0–0.5 M KCl linear gradient in TDEG buffer at a flow rate of 0.5 mL/min. The flow-through and initial fractions containing the Ap_4A binding activity were concentrated to 30 mL by using an Omegacell concentrator (fraction V).

(F) Superose-12 FPLC Column. Fraction V was applied to a 100-mL Superose-12 FPLC gel filtration column (Pharmacia). For each round of gel filtration, 2 mL of fraction V was used, and gel filtration was performed at room temperature. The column was equilibrated and developed with TDEG buffer containing 100 mM KCl at a flow rate of 2 mL/min. An aliquot of each fraction was assayed for Ap_4A binding activity, and peak fractions were pooled for further characterization.

Assay of Ap_4A Binding Activity. For Ap_4A binding assays, a 20-μL sample was incubated for 1 h in a 100-μL reaction volume consisting of 100 mM sucrose/30 mM Hepes (pH 7.8/5 mM $MgCl_2$/0.5 mM ATP/1 μM Ap_4A/0.05 mg of heated BSA/0.05 μCi of [^{3}H]Ap_4A (6.2 Ci/mmol, Amersham). After incubation, 50 μL of a suspension of acid-washed activated charcoal (100 mg/mL in water) was added to the reaction mixture. The contents of the tube were then mixed by a vortex for 10 s and kept on ice for 10 min. The charcoal was removed by centrifugation at full speed in an Eppendorf microcentrifuge. One hundred microliters of supernatant was used for determining the radioactivity in a liquid scintillation counter. Because some free [^{3}H]Ap_4A was resistant to charcoal absorption, a blank tube without sample was used as a control, and its background radioactivity was subtracted from the sample radioactivity. In cases where Triton X-100 was present in samples, Triton X-100 concentration was determined for each fraction (as described below), and the same detergent concentration was included in a blank tube as control.

Polyacrylamide Gel Electrophoresis. Gradient SDS–polyacrylamide gel (3% stacking gel and 4–15% gradient separating gel) was formed and electrophoresed at 150 V for 4 h (Laemmli, 1970). Prestained low molecular weight markers (Bio-Rad Corp.) were run on the same gel as marker proteins. Native polyacrylamide gel (3% stacking gel and 4–15% linear gradient separating gel) was electrophoresed at 4 °C in the absence of SDS. The native gel was preelectrophoresed for 1 h, and then samples were electrophoresed at 150 V for 16 h. The gels were fixed and stained with silver reagent by the procedure of Morrissey (1981).

Estimation of Triton X-100. The amount of Triton X-100 (Bio-Rad) in the sample was estimated (Horigome & Sugano, 1983) so that the effect of Triton X-100 on the binding activity could be assessed. To measure the amount of the detergent in the sample, 100 μL of sample was mixed with 400 μL of acetone/2-propanol (v/v 1:1). The mixture was incubated at 30 °C for 30 min and then centrifuged for 10 min at full speed in an Eppendorf microcentrifuge. The supernatant was removed, and its absorbance at 275 nm was determined in a Beckman DU-64 spectrophotometer. The absorbance reading was then compared to a standard absorbance curve generated with various known amounts of Triton X-100 (Horigome & Sugano, 1983).

Protein Cross-Linking Studies. The noncleavable, amine-reactive homobifunctional cross-linker disuccinimidyl suberate (DSS, Pierce) was used for cross-linking subunits of the Ap_4A binding protein. DSS was prepared fresh as a 50 mM stock in dimethyl sulfoxide. Purified Ap_4A binding protein was incubated with a series of increasing DSS concentrations in a total volume of 40 μL and incubated on ice for 30 min. The concentration of dimethyl sulfoxide was kept constant in all tubes. The reaction was terminated by addition of 4× SDS–PAGE sample buffer, heated at 100 °C for 10 min, and then applied to an SDS–PAGE gel, as described above. Both high and low molecular weight markers were used on the same gel for comparison.

Electroblotting and Amino Acid Composition Analysis. To prepare the sample for amino acid analysis, purified Ap_4A binding protein was subjected to SDS–PAGE. Proteins in the gel were transferred to an Immobilon (PVDF, Millipore) membrane as described previously (Jindal & Vishwanatha,

Table I: Summary of Ap_4A Binding Protein Purification[a]

purification step	volume (mL)	[protein] (mg/mL)	activity[b] total (units)	activity[b] specific (units/mg)	purification (x-fold)
crude extract	300	10.8	205100	64	1
DNA–cellulose	150	4.1	430500	700	10.9
DEAE Bio-Gel	200	0.98	282240	1440	22.5
butyl-Sepharose	150	0.52	129236	2105	32.9
Mono-Q	100	0.62	262841	4239	66.2
Superose-12	80	0.28	189325	8542	132

[a] The starting material was 50-g wet weight of HeLa cells growing in suspension culture. [b] One unit represents binding of 1 pmol of Ap_4A to Ap_4A bp in 1 h at 30 °C.

1990). The transfer was performed at 150 mA per gel for 90 min (Matsudaira, 1987). Proteins in the Immobilon were visualized by staining the membrane with 0.5% Coomassie brilliant blue in 50% methanol for 10 min and then destained with 50% methanol and 10% acetic acid for 5 min. The membrane was blot-dried, and areas of the membrane corresponding to Ap_4A binding protein subunits were excised from the membrane and sliced into small pieces. One hundred microliters of extraction buffer (75% 2-propanol/5% trifluoroacetic acid) was added, and the tube was incubated overnight at 65 °C. After incubation, the supernatant extract was removed to a fresh tube, and the membrane pieces were reextracted with another 100 μL of the extraction buffer for 1 h at 65 °C. The two supernatant extracts were combined and concentrated to 60 μL. The sample was then subjected to acid hydrolysis. The amino acid composition was determined on a Beckman 6300 amino acid analyzer.

Results

Resolution of Ap_4A Binding Protein from Pol α_2 Complex. Ap_4A binding activity copurifies with pol α_2 complex through the first four chromatographic columns: DE-52, native DNA–cellulose, denatured DNA–cellulose, and DEAE Bio-Gel A. Upon DEAE Bio-Gel A chromatography, pol α activity can be resolved into three forms designated as α_1, α_2, and α_3 (Lamothe et al., 1981; Vishwanatha et al., 1986). Pol α_2 containing the Ap_4A binding protein is the multiprotein form and is eluted with buffer TDEG/0.15 M KCl (Vishwanatha et al., 1986). Pol α_2 is the primase-associated bona fide DNA polymerase α. Ap_4A binding activity and pol α activity, however, were separated by hydrophobic interaction chromatography on butyl-Sepharose. The bulk of Ap_4A binding activity was eluted with TDE buffer containing 0.5% Triton X-100, indicating the highly hydrophobic nature of the binding protein. All of pol α–primase activity was eluted with TDE buffer without detergent. Only a minor amount of Ap_4A binding activity was found in the TDA wash. The major binding activity eluted from the column with detergent was used for further purification.

Purification of Ap_4A Binding Protein to Homogeneity. Ap_4A binding protein resolved from pol α–primase on the butyl-Sepharose column was purified to homogeneity. Table I summarizes data from one typical HeLa cell fractionation for purification of Ap_4A binding activity. The purified Ap_4A binding protein has a specific activity of 8452 units/mg, and a purification of 132-fold was obtained. Homogeneously purified binding protein was obtained from pol α_2 by chromatography on butyl-Sepharose, Mono-Q FPLC, and gel filtration on Superose-12 FPLC. The total activity fluctuates in the various chromatographic steps. In the crude extracts, a

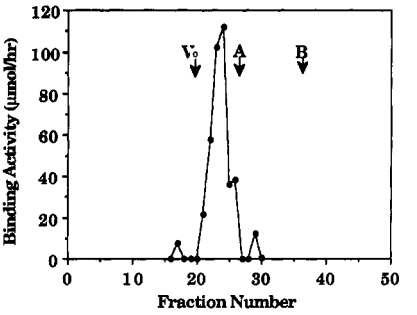

FIGURE 1: Gel filtration of Ap_4A binding protein on a Superose-12 FPLC column. Ap_4A binding protein (2 mL) from the Mono-Q FPLC column (Table I) was subjected to gel filtration chromatography over a 100-mL preparative Superose-12 FPLC column equilibrated and developed in TDE buffer containing 50 mM KCl. Fractions of 2 mL were collected, and an aliquot of each fraction was assayed for Ap_4A binding activity. The void volume (V_0) and positions of the marker proteins aldolase (A, 158 kDa) and cytochrome c (B, 13 kDa) are indicated.

number of Ap_4A phosphohydrolases are present, and thus the total activity in the crude extract is an underestimation of the Ap_4A binding activity. We do not know the reason for low activity in the butyl-Sepharose step and the increase in activity in the following Mono-Q step. Perhaps an inhibitor of binding activity present during butyl-Sepharose chromatography is removed during Mono-Q chromatography. We subjected fraction IV to chromatography on the Mono-Q FPLC column. While the Ap_4A binding protein remains unbound to this column, this chromatographic step was helpful in removing a vast majority of contaminating proteins, which remained bound to the Mono-Q column. The unbound fraction from the Mono-Q column was concentrated and subjected to gel filtration on the Superose-12 FPLC preparative column (100-mL volume). This gel filtration column served as the final step for the purification of the Ap_4A binding protein. In our pilot experiments, we found that inclusion of 100 mM KCl in TDEG buffer improved resolution of the Ap_4A binding activity. Ap_4A binding activity elutes as a single peak centered around an elution volume of 48 mL (Figure 1). The apparent native molecular weight, according to a plot of log molecular weight against V_e/V_0, was calculated to be 200 000. The purified binding activity at this stage is stable for over 3 months and is unaffected by freezing and thawing.

Subunit Composition of Ap_4A Binding Protein. Aliquots of fractions collected from the Superose-12 gel filtration column (Figure 1) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by silver staining as described under Experimental Procedures. Figure 2A shows that the Ap_4A binding activity is contributed by two polypeptides of 45 000 and 22 000 Da. In these fractions, densitometric analysis indicates that greater than 95% of the protein loaded in each lane is represented by the two polypeptides seen. We designate the 45 000-Da polypeptide as A_1 and the 22 000-Da polypeptide as A_2. Upon electrophoresis of the Superose-12 gel filtration fractions under nondenaturing conditions, Ap_4A binding activity migrates as a single complex of 200 000 Da (Figure 2B). The molecular weight of this native form of Ap_4A binding protein determined by gel electrophoresis is consistent with that calculated by gel filtration (Figure 1). The densitometric intensity of the bands on the gel correlates well with the binding activity (Figure 1). We have been unable to recover the binding activity from the native PAGE under a variety of elution conditions as the eluted protein has no binding activity. The association of A_1 and A_2 subunits in the native complex was further substantiated by cross-linking the two subunits using the noncleavable,

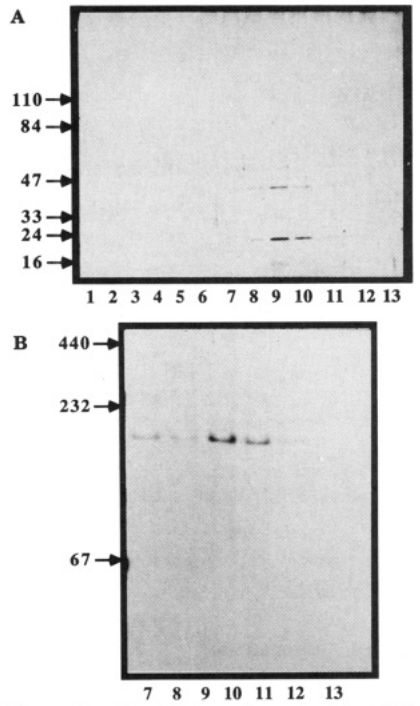

FIGURE 2: Polyacrylamide gel electrophoresis of Ap_4A binding protein. Various indicated fractions from the Superose-12 gel filtration column included in the peak of binding activity were subjected to electrophoresis on 4–15% gradient polyacrylamide gels either under denaturing conditions (panel A) or under nondenaturing conditions (panel B). The gels were fixed and stained with silver nitrate. Positions of the appropriate markers run on the same gels are indicated with their molecular masses in kilodaltons. Lanes 1–13 represent fractions 15–27 from the Superose-12 gel filtration (Figure 1).

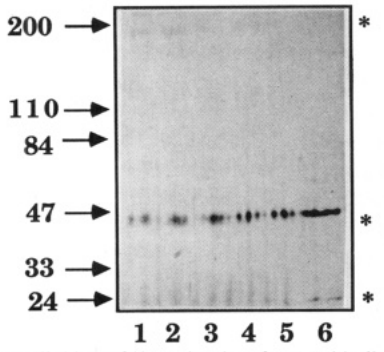

FIGURE 3: Cross-linking of the subunits of Ap_4A binding protein with DSS. Purified Ap_4A binding protein was treated with the protein cross-linking reagent DSS (disuccinimidyl suberate, Pierce) as described under Experimental Procedures. Lane 6 is in the absence of cross-linker, and lanes 1–5 represent DSS concentrations of 50, 20, 10, 5, and 2.5 mM. Positions of the two subunits and the cross-linked protein bands are indicated by asterisks.

amine-reactive homobifunctional cross-linker DSS. DSS has been used in coupling the A and B chains of ricin (Montesano et al., 1982) and in cross-linking many ligands to their receptors. As shown in Figure 3, with increasing concentrations of DSS, A_1 and A_2 polypeptides are cross-linked to generate a higher molecular weight complex on SDS–PAGE (indicated by an asterisk in Figure 3). The cross-linked high molecular weight complex cannot be disrupted by treatments usually used to separate noncovalently bonded peptides, such as heating at 100 °C and SDS–PAGE. The cross-linked complex migrates as a 210000-Da protein. This is in complete agreement with the native molecular weight determined on the Superose-12 gel filtration column (Figure 1) and by native gel electrophoresis (Figure 2B). We do not know the exact stoichiometry of the A_1 and A_2 subunits in the native complex.

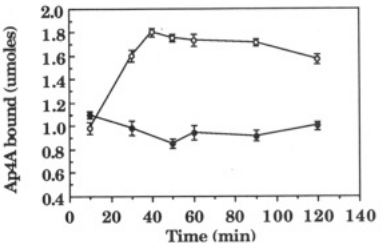

FIGURE 4: Time course of Ap_4A binding to the binding protein. Binding of [^{3}H]Ap_4A to the purified binding protein was monitored as a function of time. Open circles indicate Ap_4A binding by the purified protein, and closed circles represent TDE/Triton buffer alone.

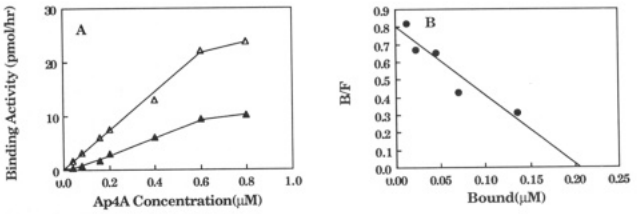

FIGURE 5: Analysis of Ap_4A binding to the binding protein. Purified binding proteins from the Superose-12 gel filtration column were used in this assay. Protein concentration was 40 μg/mL. Binding assay was carried out as described under Experimental Procedures. Panel A represents the binding activity as a function of Ap_4A concentration in the presence of binding protein (open symbols) or TDE/Triton X-100 buffer alone (closed symbols). Panel B represents the Scatchard analysis of Ap_4A binding to Ap_4A binding protein. *B* and *F* are micromoles of Ap_4A bound to the Ap_4A binding protein and free Ap_4A, respectively. Free Ap_4A concentration was obtained by subtracting *B* from the total ligand concentration. Values for bound ligand concentration are from panel A. Scatchard analysis gives a K_D of 0.25 μM.

Absence of Contaminating or Associated Enzymatic Activities. We have analyzed the purified Ap_4A binding protein for various enzymatic activities that might affect DNA synthesis. The purified Ap_4A binding protein lacks detectable pol α, pol β and δ, DNA primase, deoxyribonuclease, DNA-dependent or DNA-independent ATPase, topoisomerase, RNase H, or DNA ligase activities.

Characterization of Ap_4A Binding Activity. The purified Ap_4A binding protein specifically binds to Ap_4A, and this specific binding is unaffected by the presence of a vast excess of a structurally related compound such as ATP. In the routine Ap_4A binding assay, 1 μM Ap_4A and 500 μM ATP are included. Further, the binding activity is unaffected by inclusion of other NTPs or dNTPs in the reaction. Ap_4A binding protein does not bind to radiolabeled NTPs and dNTPs (data not shown). The binding activity is not dependent on the presence of divalent metal ions in the reaction. The time course of binding to Ap_4A is shown in Figure 4.

The presence of Triton X-100 was essential for maintaining the stability and activity of the binding protein. Removal of the detergent by chromatography on Extracti-gel (Pierce) results in complete loss of activity. However, Triton X-100 interfers with the binding activity. In order to obtain accurate binding data, the amount of detergent in each sample was estimated, and the same amount of Triton X-100 was included in the blank tube for determination of background activity.

The dissociation constant of the Ap_4A binding protein–[^{3}H]Ap_4A complex has been calculated by the charcoal adsorption method to be 0.25 μM (Figure 5) when analyzed by means of a Scatchard plot (Scatchard, 1949).

Amino Acid Composition of Ap_4A Binding Protein. The purified Ap_4A binding protein was subjected to amino acid composition analysis according to procedures detailed under Experimental Procedures, and the results are presented in

Table II: Amino Acid Composition of Ap_4A Binding Protein[a]

	mol of AA/mol of protein			
	A_1		A_2	
amino acid	1	2	1	2
Asp	43.4	40.0	18.8	19.3
Thr	26.6	21.7	6.9	10.7
Ser	55.6	44.4	18.0	24.0
Glu	52.0	51.8	28.5	27.5
Pro	21.1	9.6	6.1	7.4
Gly	24.9	22.8	38.0	39.1
Ala	25.5	28.9	16.4	14.6
Cys	ND[b]	ND	ND	ND
Val	20.3	21.2	10.5	11.3
Met	4.0	7.0	1.5	1.5
Ile	13.1	20.1	5.0	6.0
Leu	29.1	33.2	20.1	17.1
Tyr	15.0	14.2	5.1	7.4
Phe	10.9	12.5	5.9	7.1
His	6.3	7.7	3.3	4.0
Lys	14.0	20.8	12.3	8.8
Arg	20.3	16.2	10.3	6.8

[a] Amino acid compositions from two separate determinations are presented. Values are normalized to molecular weights of 45 000 for A_1 and 22 000 for A_2. [b] ND, not determined.

Table II. The two subunits differ in their amino acid composition. Both subunits are characterized by approximately equal distribution of nonpolar, polar uncharged, and charged amino acids. The acidic amino acid content is higher than basic amino acids for both subunits. The hydrophobicity of the binding protein is reflected by the presence of hydrophobic amino acids at approximately a third of the total amino acid residues.

Discussion

Ap_4A has been proposed to be involved in multiple cellular processes such as DNA replication, DNA repair, cell proliferation, platelet aggregation, and vascular tonus (Andersson, 1989). A protein that specifically binds to Ap_4A is found in tight association with DNA pol α from calf thymus, HeLa cells, and *Xenopus Laevis* oocytes (Baril et al., 1983; Rapaport & Feldman, 1984; Zourgui et al., 1988). The binding of Ap_4A to this protein may modulate the process of DNA replication. In this respect, it is interesting that poly(ADP-ribosylated) Ap_4A inhibits SV40 DNA replication in vitro (Baker et al., 1987). We have devised a protocol for purification of Ap_4A binding protein from human HeLa cells to homogeneity, and using the purified protein, we have characterized the binding activity. The Ap_4A binding protein is hydrophobic in nature and is separable from the pol α complex (Vishwanatha et al., 1986) upon butyl-Sepharose chromatography. At this stage of purification, the fraction containing the binding activity is highly impure. Further chromatography on Mono-Q FPLC and Superose-12 FPLC columns results in complete purification of the binding protein.

The purified binding protein has a mass of 200 kDa in the native state as determined by gel filtration chromatography and by nondenaturing polyacrylamide gel electrophoresis. The binding protein resolves into two polypeptides of 45 and 22 kDa upon SDS–PAGE. We designated these two polypeptides as A_1 and A_2, respectively. The two subunits can be cross-linked using DSS, and the resulting complex has a mass of 210 kDa on SDS–PAGE in agreement with the native size of the binding protein. The purified protein is free of contaminating enzymatic activities. The subunit composition determined by us is in disagreement with previously published data on Ap_4A binding protein from HeLa cells (Baril et al., 1983), where the binding protein was found to be a single subunit of 47 kDa. We do not know the reason for this discrepancy.

The binding activity is specific for Ap_4A, and a dissociation constant of 0.25 μM was determined by Scatchard analysis. This indicates a high affinity of Ap_4A to Ap_4A binding protein. The dissociation constant of homogeneously purified HeLa Ap_4A binding protein is considerably less than the dissociation constant of 38 μM for the binding protein from *Xenopus laevis* oocytes (Zourgui et al., 1988) and 13 μM for the calf thymus (Rapaport & Feldman, 1984) Ap_4A binding protein. The binding proteins from these sources have different subunit compositions and combined with the differences in dissociation constants may be a reflection of multiple Ap_4A binding activities in the cell. The amino acid composition of Ap_4A binding protein reflects the hydrophobicity of this protein. Nonpolar amino acids constitute approximately a third of the total amino acid residues in both the subunits.

The physiological role of Ap_4A binding protein in eukaryotic cells has not been established. In view of the multiple roles of Ap_4A in cells, it will be interesting to determine the role of the binding protein in these cellular processes. With the availability of homogeneous Ap_4A binding protein preparations, it should be possible to define the physiological role of this protein.

Registry No. pol α, 9012-90-2.

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