Characterization of the HeLa Cell DNA Polymerase α-Associated Ap_4A Binding Protein by Photoaffinity Labeling†

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ABSTRACT: The ubiquitous dinucleotide diadenosine 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 to Ap_4A is associated with a multiprotein form of DNA polymerase α (pol α_2) in HeLa cells. We have purified the pol α-associated Ap_4A binding protein to homogeneity. The Ap_4A binding protein is resolved into two polypeptides of 45 and 22 kDa, designated as A_1 and A_2, respectively. We have utilized [α-^{32}P]8-N_3-Ap_4A to label the purified binding protein, and by cross-linking the photoaffinity label we have determined that Ap_4A binds to the A_1 subunit. No binding to the ligand is observed with the A_2 subunit. Photoaffinity labeling is saturated with approximately 0.4 μM photolabel, with a half-maximal binding at 0.15 μM. The labeling is UV-dependent and is competed by both 8-N_3-Ap_4A and Ap_4A. Photoaffinity labeling is not affected in the presence of dATP and dGTP and is reduced only in the presence of excess of ATP indicating the specificity of the protein for Ap_4A. Of the diadenosine polyphosphates, Ap_4A and Ap_5A competed for binding, while Ap_2A and Ap_3A did not compete for binding. Further, the presence of at least one adenosine may be necessary since Ap_4G competes but Gp_4G does not compete for binding to the protein. Various methylene bisphosphonate and thiophosphate analogs of Ap_4A were tested to see their effect on photoaffinity labeling with 8-N_3-Ap_4A. Significant differences were observed among the various analogs in their ability to prevent the photoaffinity labeling of the ligand to the binding protein.

Diadenosine 5′, 5‴-P^1,P^4-tetraphosphate (Ap_4A)[1] is present in prokaryotes and eukaryotes. Ap_4A has diverse roles in cellular metabolism. Intracellularly, it has been proposed to have a role in DNA replication, DNA repair, and cell proliferation. Recent studies have called attention to the extracellular, intravascular, and possibly other transmitter activities of Ap_4A and related dinucleotides (McLennan & Zamecnik, 1992). Intracellular levels of Ap_4A are at basal levels of 10^{-8} to 10^{-6} M. The level of Ap_4A in cells has been shown to be correlated with cellular proliferative state. Its levels also increase when cell are stressed by heat shock, oxidative stress, etc.

A role for Ap_4A in cell proliferation and DNA replication was originally proposed on the basis of observations that Ap_4A intracellular levels increase with cellular proliferative activity (Rapaport & Zamecnik, 1976); Ap_4A induces DNA replication in permeabilized, quiescent baby hamster kidney cells (Grummt, 1978), and when microinjected into *Xenopus laevis* oocytes (Zorgui et al., 1984); Ap_4A binds to a subunit of DNA polymerase α from calf thymus (Grummt et al., 1979; Rapaport & Feldman, 1984), HeLa cells (Baril et al., 1983, Vishwanatha et al., 1986, Vishwanatha & Wei, 1992), and *X. laevis* (Zorgui et al., 1985); a change in the cellular level of Ap_4A appears to be correlated with the initiation of DNA replication in sea urchin embryos (Morioka & Shimada, 1985), and a drastic rise of intracellular Ap_4A at the G_1/S boundary of the cell cycle was reported to correlate with the onset of DNA synthesis in eukaryotes (Weinmann-Dorsch et al., 1984).

Another intracellular role proposed for Ap_4A is in the response to DNA damage and other forms of stress. The supporting evidence for this role is Ap_4A levels increase drastically under a variety of stress conditions (hyperthermia, ethanol, aromatic oxidants, sulfhydryl reagents, MNNG), which has led to the proposal that Ap_4A acts as an alarmone (Lee et al., 1983a,b; Brevet et al., 1985; Baker & Jacobson, 1986; Baker & Ames, 1988: Gilson et al., 1988). Ap_4A is a good acceptor of poly-ADP-ribose, and poly-ADP-ribosylated Ap_4A inhibits SV40 DNA replication in vitro (Baker et al., 1987). Ap_4A accumulates in cells after heat shock; after a mild heat shock, nuclear injection of Ap_4A to *X. laevis* oocytes results in marked enhancement of heat shock protein p70 expression (Guedon et al., 1985). Thus, Ap_4A is

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[1] 1Abbreviations: Ap_4A, diadenosine 5′,5‴-P^1P^4-tetraphosphate; Ap_4G, adenosine 5′-guanosine 5′-P^1,P^4-tetraphosphate; ϵAp_4A, 1,N^6-ethenoadenosine 5′-adenosine 5′-P^1,P^4-tetraphosphate; $AppCH_2ppA$, diadenosine 5′,5‴-P^1,P^4-(P^2,P^3-methylene)tetraphosphate; ApCHClpCHClpA, diadenosine 5′,5‴-P^1,P^4-(P^1,P^2-monochloromethylene-P^3,P^4-monochloromethylene) tetraphosphate; other dinucleoside polyphosphates and phosphonate analogues are abbreviated in a similar fashion; Ap_spppA, diadenosine 5′,5‴-P^1P^4-monothiotetraphosphate; Ap_sppp_sA, diadenosine 5′,5‴-P^1P^4-dithiotetraphosphate; $Ap_spCH_2pp_sA$, diadenosine 5′,5‴-P^1P^4-dithio(P^2,P^3-methylene)tetraphosphate. All sulfur-containing analogues comprised mixtures of different diastereomers.

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implicated in the regulation and possibly the termination of the heat shock response (Nover, 1986; Pelham, 1986).

An extracellular role for Ap_4A has also been proposed in platelet aggregation. Platelets are rich stores of Ap_4A (about 0.42 nmol/mg of protein, or about 1% of the ATP content), and upon thrombin-induced platelet aggregation Ap_4A is released. Ap_4A is a potent inhibitor of platelet aggregation and competitively inhibits ADP-induced platelet aggregation. The levels of Ap_4A are much lower in platelets from Chediak-Higashi syndrome (Kim et al., 1985), which may be indicative of the important physiological role of this compound. Ap_4A can disaggregate platelets in vitro, and infusion of Ap_4A in a rabbit intracarotid thrombosis model showed a reduction in the incidence of thrombosis (Louie et al., 1988). Ap_4A induces vasodilation in isolated, saline-perfused segments of rabbit mesenteric vesicles (Busse et al., 1988). Ap_4A induced a contraction in norepinephrine-treated arteries denuded of endothelium. In this respect, a specific Ap_4A receptor has recently been described in heart muscle and other tissues (Walker et al., 1993). Finally, Ap_4A and other dinucleoside polyphosphates have been proposed as novel neurotransmitters, also possibly interacting with specific dipurinergic receptors (Pintor & Miras-Portugal, 1993).

In order to elucidate the role of Ap_4A in eukaryotic DNA replication, we carried out the purification of a multiprotein DNA polymerase and associated Ap_4A binding protein from HeLa cells (Vishwanatha & Wei, 1992). In the present investigation, the purified protein was further characterized to study ligand binding by photoaffinity labeling.

EXPERIMENTAL PROCEDURES

Growth of HeLa cells, fractionation, and purification of Ap_4A binding protein was carried out as described previously by Vishwanatha and Wei (1992). In brief, the cells were grown in Joklik's modification of minimal essential medium supplemented with fetal calf serum. A 1000-mL culture of cells at 10^6 cells/mL density was harvested and Dounce homogenized. The mitochondrial and microsomal fractions were separated from cytoplasmic fraction. The nuclear pellet was treated with nuclear extract buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, and 0.15 M KCl) on a nutator for 1 h at 4 °C. The nuclear extract was then centrifuged at 45 000 rpm for 1 h at 4 °C in a Beckman Ti60 rotor. The supernatant comprising nuclear extract was combined with cytoplasmic extract. The crude extract thus obtained was fractionated on DEAE-cellulose (DE-52) anion exchange column, single-stranded and double-stranded DNA-cellulose columns, DEAE-Bio-Gel A, butyl-Sepharose, Mono-Q FPLC, and finally a Superose-12 FPLC column as described earlier. At each step of purification pol α activity was assayed (Vishwanatha et al., 1986). The Superose-12 FPLC fractions containing Ap_4A binding activity were pooled, concentrated, and used for further labeling experiments.

Synthesis of Photoaffinity Probe 8-N_3-Ap_4A. 8-N_3-Ap_4A was synthesized as per the method of Prescott and McLennan (1990). All the operations involving photoaffinity label (N_3) were carried out in subdued light. Briefly, the synthesis involves mixing of 1 μL of 0.3 M $MgCl_2$, 3 μL of 0.1 M 8-N_3-AMP (Sigma Chemical Co., St. Louis, MO), and 250 μCi of [α-^{32}P]ATP (specific activity 3000 Ci/mmol, ICN Biochemicals, Irvine, CA) and evaporation to dryness in a Speedvac. Subsequently, 5 μL of a 1:1 mixture of 3.4 M Hepes NaOH, pH 6.8, and 5 M carbodiimide (Sigma Chemical Co., St. Louis, MO), prepared just before use, was added to the bottom of the tube, and the residues were carefully dissolved. The mixture was incubated in dark at 37 °C in a waterbath for 3 h. After 3 h, 400 μL of 0.05 M triethyl ammonium bicarbonate (TEAB) buffer, pH 8.6, (Sigma Chemical Co., St. Louis, MO) was added to terminate the reaction. The reaction product was injected onto a Mono-Q FPLC (0.5 cm × 5 cm, Pharmacia) column at a flow rate of 0.5 mL/min. The column was washed with 0.05 M TEAB, pH 8.6, to remove the unbound material of the reaction mixture. The bound nucleotides were eluted with a 20-mL linear gradient of 0.05−0.7 M TEAB, pH 8.6, at a flow rate of 0.5 mL/min, and the column elution was monitored in a ultraviolet wavelength detector at 254 nm. Fractions of 1 mL were collected and from each fraction; a 1-μL aliquot was used to determine radioactivity in a scintillation counter. Fractions containing [α-^{32}P]8-N_3-Ap_4A were combined and concentrated by Speedvac. The residues were dissolved in 100 μL of Milli-Q H_2O. Furthermore, the presence of 8-N_3-Ap_4A was qualitatively characterized by thin layer chromatography using butyric acid/H_2O/NH_4OH (66:33:1) as a solvent system (Salvucci et al., 1992). The purity of the 8-N_3-Ap_4A was routinely checked by thin layer chromatography using mononucleotides and dinucleoside polyphosphate as standards. Ap_4G, Gp_4G, ϵAppppA, and Ap_spppA were prepared and characterized by the above procedures using appropriate substrates. $AppCH_2ppA$ was prepared as previously described (Taylor, 1988). All other Ap_4A analogs were kindly provided by G. M. Blackburn, G. E. Taylor, and M.-J. Guo, University of Sheffield, U.K.

Photoaffinity Labeling of Protein. Photoaffinity labeling of protein was carried out in a final volume of 50 μL containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and an appropriate amount of [α-^{32}P]8-N_3-Ap_4A. The reaction mix was incubated in the dark at 37 °C for 1 h, followed by incubation at 0−4 °C on ice for 5−10 min in the inverted cap of a microcentrifuge tube (in the form of a thin uniform film). Photolysis was performed by exposure to short wavelength ultraviolet light at 254 nm for 120 s using a Stratalinker (Stratagene, La Jolla, CA). At the surface of the protein film, an energy dose of 3300 μJ/(m^2·s) was achieved. Nonphotolyzed controls samples were kept unexposed on ice. The reaction mixture was quenched by addition of SDS sample buffer (250 mM Tris-HCl, pH 6.8, 10% (w/v) β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). It was then heated at 95 °C for 5 min. The sample was loaded onto a 10% denaturing polyacrylamide gel. Electrophoresis was carried out at 150 V for 5 h. Following electrophoresis, the gel was dried under vacuum. The dry gel was scanned for radioactivity in a Betascope 603 (Betagen, Waltham, MA) radioanalytical imager and exposed to Kodak X-Omat film using a Cronex intensifying screen (DuPont-NEN, Boston, MA).

High-Performance Electrophoretic Chromatography. High-performance electrophoretic chromatography (Applied Biosystem, Inc., Foster City, CA) of Ap_4A binding protein was carried out as per manufacturer's instructions. In principle, the Superdex-75 FPLC fraction containing the Ap_4A binding protein was cross-linked to [α^{32}P]8-N_3-Ap_4A and quenched with SDS buffer as described above. The quenched sample

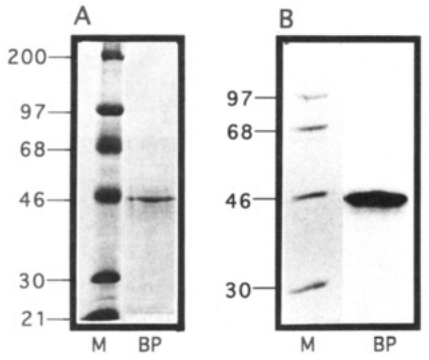

FIGURE 1: Purification and labeling of the protein. HeLa cell Ap_4A binding protein was purified to homogeneity. The purified protein was subjected to labeling with 4.8 nM [α^{32}P]8-N_3-Ap_4A (specific activity: 4×10^5 cpm/pmol) in a total volume of 50 μL. One aliquot was subjected to SDS−PAGE followed by silver staining (panel A), and another aliquot was subjected to SDS−PAGE followed by autoradiography (panel B). Lane M refers to ^{14}C-labeled molecular weight markers whose sizes are indicated on the *y* axis. Lane BP represents the purified binding protein.

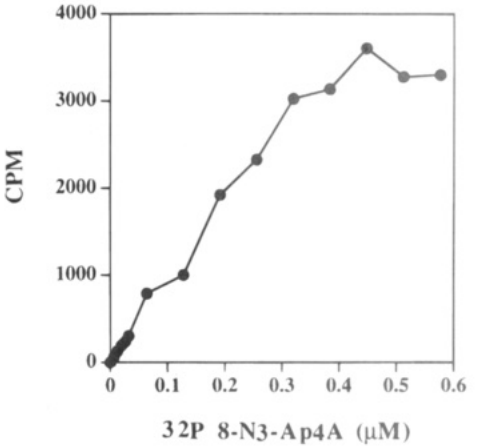

FIGURE 2: Saturability of photolabeling with 8-N_3-Ap_4A. Photoaffinity labeling reactions were set up with purified binding protein and various indicated concentrations of 8-N_3-Ap_4A. The specific activity of radiolabeled photolabel was kept constant (4×10^5 cpm/pmol) at the different substrate concentrations. Following gel electrophoresis, the radioactive band was quantitated on a Betascope 603, and the data are indicated as cpm in cross-linked protein band as a function of substrate concentration.

was kept at 95 °C for 5 min. The sample was then loaded onto an 8% denaturing polyacrylamide tube gel (2.5 mm × 100 mm). A linear current gradient of 0.4−0.8 mA was applied for the initial 10 min for the sample to penetrate the gel. A continuous current of 0.8 mA was then applied for 500 min at 5 °C. Elution of the proteins from the gel was done with 0.25 M Tris-HCl, pH 8.3, at 280 nm, 0.1 absorption units. Fractions were collected by peak detection above 5% threshold. The fractions were then analyzed for radioactivity by adding 1-μL aliquots to 5 mL of Ecolume. Fractions containing peak radioactivity in the protein eluting region were further concentrated and analyzed for the cross-linked Ap_4A binding protein by electrophoresis on a 10% denaturing polyacrylamide gel.

RESULTS

Purification of Ap_4A Binding Protein to Homogeneity. Purification of an Ap_4A binding protein associated with HeLa cell DNA polymerase α and demonstration of [^{3}H]Ap_4A binding activity has been reported earlier (Vishwanatha & Wei, 1992). The purified binding protein was found to have two polypeptides of 22 and 45 kDa. In the present studies we carried out further purification of these two polypeptides using high-performance chromatographic electrophoresis. Characterization of the 45-kDa subunit by photoaffinity labeling of Ap_4A is presented below.

Photoaffinity Labeling of Ap_4A Binding Subunit. In order to determine which of the two subunits binds to Ap_4A, we used photoaffinity labeling of the binding protein to [α-^{32}P]8-N_3-Ap_4A. This ligand is a high specific activity substrate with a photoactive azido group. When the complete Ap_4A binding protein (consisting of the two subunits) was used in photolabeling, only the 45-kDa subunit bound the photolabel and there was no photolabeling of the 22-kDa subunit (data not shown). Purified 45-kDa subunit binds to the photolabel (Figure 1). Thus, the subunit of the Ap_4A binding protein that binds to the ligand is the 45-kDa subunit. The role of the smaller 22-kDa subunit in the binding activity is not known. The labeling of 8-N_3-Ap_4A to the 45-kDa subunit is dependent upon exposure to UV. This is evident from the absence of a radioactive band in unexposed sample (lane U in Figures 3 and 7). Furthermore, the design of the [α-^{32}P]8-N_3-Ap_4A is such that no likely degradation products can contain both radiolabel and photoactivable azido group, thus ensuring that any labeling is due to the intact molecule. To reduce nonspecific binding of the radiolabel, we included 50 nM ATP (10-fold excess over radiolabel) in the reaction mixture. During the course of these studies it was noted that labeling is abolished by the presence of dithiothreitol and that photolabeling was qualitatively similar when either Hepes-NaOH, pH 7.8, or Tris-HCl, pH 7.5, buffer was used (data not shown).

The presence of the photoaffinity cross-linked 45-kDa band was monitored in various chromatographic fractions during the purification procedure. Purified 45-kDa protein was used in all the further experiments. Experiments were carried out on the effect of various concentrations of ligand on the labeling to determine binding saturation (Figure 2). Varying concentrations of [α-^{32}P]8-N_3-Ap_4A (specific activity: 4×10^5 cpm/pmol) in the range of 0−0.6 μM were used. At a concentration of about 0.4−0.5 μM [α-^{32}P]8-N_3-Ap_4A, labeling became saturated with half-maximal labeling at approximately 0.15 μM. Photolabeling was linear with increasing protein concentrations (data not shown). In another set of experiments (Figure 3), we determined the specificity of photolabeling by incubating 4.8 nM [α-^{32}P]8-N_3-Ap_4A (specific activity: 4×10^5 cpm/pmol) with increasing concentrations of unlabeled 8-N_3-Ap_4A (Figure 3, panels B and C) or unlabeled Ap_4A (Figure 3, panels A and D). Photolabeling was reduced with increasing concentrations of 8-N_3-Ap_4A and Ap_4A. Inclusion of unlabeled 8-N_3-Ap_4A along with 4.8 nM [α-^{32}P]8-N_3-Ap_4A resulted in diminished photolabeling (Figure 3, panel C) which is a consequence of diluting the radiolabel. The specificity of photolabeling was confirmed by lack of inhibition of photolabeling in the presence of N_3-ATP at concentrations 10−100-fold higher than the concentrations of 8-N_3-Ap_4A used in Figure 3C (data not shown). It was noted that Ap_4A (without the azido group) efficiently competed the photolabeling reaction with a 50% reduction in labeling in the presence of 1 to 5 μM Ap_4A (Figure 3, panel D). Between

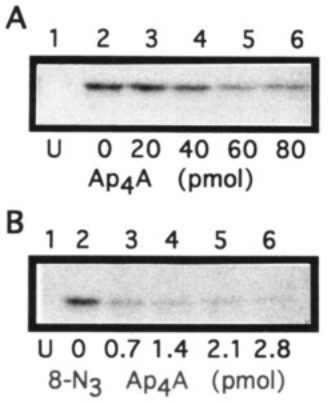

FIGURE 3: Competition for labeling with 8-N_3-Ap_4A and Ap_4A. Photoaffinity labeling experiments were set up as described under Experimental Procedures. Indicated concentrations (pmols/50 μL reaction mix in panels A and B) of unlabeled 8-N_3-Ap_4A and Ap_4A were added to the reaction. Panels A and B show autoradiograms of one experiment with 8-N_3-Ap_4A (panel B) or Ap_4A (panel A). Lane U represents the result of no UV labeling. Panels C and D show composites of several experiments showing a gradual decrease in binding activity with added nonradioactive 8-N_3-Ap_4A (panel C) or Ap_4A (panel D), as indicated.

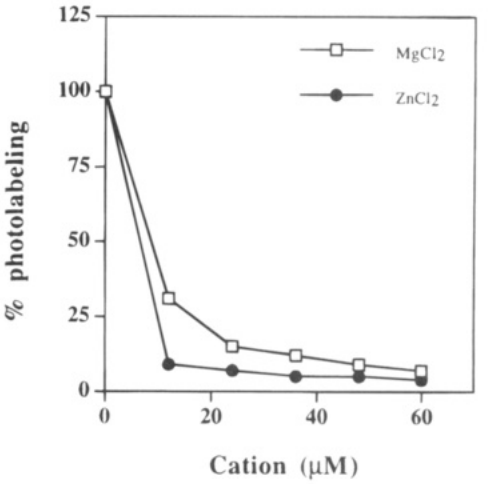

FIGURE 4: Effect of divalent cations on labeling. Photoaffinity labeling experiment was set up as described under Experimental Procedures. Indicated concentrations of $MgCl_2$ and $ZnCl_2$ were added to the reaction. The effect of increasing concentrations of the cations on the photolabeling reaction is shown.

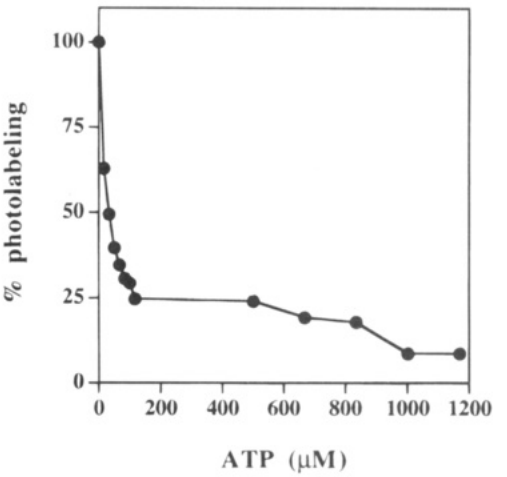

FIGURE 5: ATP at high concentrations reduces photolabeling. Various concentrations of ATP were added to the photolabeling reaction set up as described under Experimental Procedures. The extent of photolabeling was monitored by scanning the dry gel on a Betascope. The effect of various indicated concentrations of ATP on photolabeling is shown as a percent of control photolabeling in the absence of any added ATP.

1 and 5 μM Ap_4A, no differences were observed in extent of reduction in photolabeling. With increased Ap_4A concentrations (50 μM and above), photolabeling was severely reduced. Higher concentrations of Ap_4A were needed for competing photolabeling than 8-N_3-Ap_4A. A similar observation was made by Pomerantz et al. (1975) who found that an excess of cAMP was required to half-maximally inhibit the photoincorporation of 8-N_3-cAMP into a protein kinase from bovine brain. The higher concentration of nonphotoactive ligand required to inhibit photolabeling can be explained on the basis of the reversible binding of the nonphotoactive ligand and the covalent binding of the photoactive ligand (Pomerantz et al., 1975). In reversible binding, the ratio of Ap_4A bound to 8-N_3-Ap_4A bound will be determined by their affinities and concentrations. Since the binding of these two ligands to the 45-kDa subunit is presumed to be in dynamic equilibrium, there will be continuous exchange of the nonphotoactive and photoactive ligands. In contrast, during covalent binding of 8-N_3-Ap_4A, there will be a trapping effect, whereby 8-N_3-Ap_4A molecules which exchange with [α-^{32}P]8-N_3-Ap_4A molecules during UV exposure can be covalently incorporated. This will be reflected in a higher apparent affinity for covalent binding than for noncovalent binding. Thus, higher concentration of Ap_4A than 8-N_3-Ap_4A will be needed for reducing photolabeling.

Effect of Divalent Cations on Photolabeling. The role of divalent metal ions and their requirement both in prokaryotes and eukaryotes for the interaction of Ap_4A with proteins, especially the hydrolytic enzyme Np_4Nase, have been well studied (Guranowski & Sillero, 1992). It was of interest to see the effect of Mg^{2+} and Zn^{2+} on the affinity labeling to the 45-kDa protein. Figure 4 shows that both Zn^{2+} and Mg^{2+} caused inhibition of labeling. At 10 μM concentration of cations, there was a significant reduction in labeling. Presence or absence of 1 mM EDTA in the labeling reaction had no effect on the inhibition of photolabeling by Zn^{2+} and Mg^{2+} (data not shown). This reduction in affinity labeling by Mg^{2+} and Zn^{2+} may be an effect of cations on the conformation of the ligand resulting in lack of binding to the Ap_4A binding protein.

Effect of Nucleotides on Photolabeling. In order to have a better understanding of the specificity of Ap_4A binding to the 45-kDa protein, it is necessary to determine the effect of some Ap_4A-related compounds on ligand binding. NTP, dNTPs, and Np_nN can be effectively used in such studies. We used ATP, dATP, dGTP, Ap_4G, Gp_4G, and diadenosine polyphosphates such as Ap_2A, Ap_3A, and Ap_5A. Figure 5 shows the effect of increasing ATP on the labeling of [α-^{32}P]8-N_3-Ap_4A. At a concentration of 10 μM ATP (2000-fold excess over [α-^{32}P]8-N_3-Ap_4A), photolabeling was only reduced by 20%. There was a significant inhibition of photolabeling caused by ATP at higher concentrations (50% inhibition at 50 μM ATP). However, this inhibition results from the fact that the concentrations of ATP are 2000−200000-fold higher when compared to the radioligand concentration (4.8 nM) used in these experiments. A similar observation was made by Grummt et al. (1979) in a binding

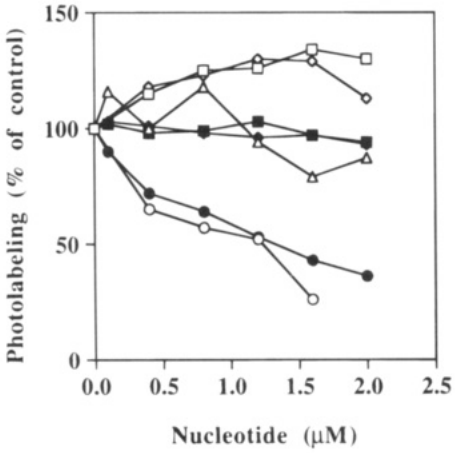

FIGURE 6: Effect of various ligands on labeling. Photoaffinity labeling experiments were set up as described under Experimental Procedures. Indicated concentrations of competing nucleotides and dinucleotides were added to the photolabeling reactions. Extent of photolabeling was monitored by scanning the dry gel on a Betascope. Data are presented as percent labeling compared to reaction with no added competitor. The various ligands used were dATP (open triangles), dGTP (closed squares), Ap_2A (open squares), Ap_3A (open diamonds), Ap_5A (open circles), Ap_4G (closed circles), and Gp_4G (closed diamonds).

reaction involving [^{3}H]Ap_4A and binding protein associated with polymerase α. This indicates that the relative affinity of the 45-kDa subunit for Ap_4A is greater than for ATP. When dATP or dGTP was used as a competitor in the labeling experiment, no significant inhibition in the binding was noted indicating that deoxynucleotides may not compete for binding (Figure 6). Our next step was to see whether Ap_nA have any competitive effect on binding of [α-^{32}P]8-N_3-Ap_4A. Ap_2A, Ap_3A, and Ap_5A were used in the experiment in varying concentrations. It was found that Ap_5A competed for binding, while Ap_2A and Ap_3A did not (Figure 6). At 1.5 μM, Ap_5A caused a 80% reduction in photolabeling.

Substitution of one of the two adenine moieties in Ap_4A by guanine to give Ap_4G did not reduce the ability of this dinucleotide to compete with 8-N_3-Ap_4A for binding (Figure 6). Reduction of photolabeling by 50% required 1.2 μM Ap_4G, which was similar to the concentrations of Ap_4A and Ap_5A required for the same 50% reduction. Gp_4G, in which both adenines are replaced by guanine, was completely ineffective as a competitor. Thus, the binding site has a high specificity for adenine.

Ap_4A Analogs and Photoaffinity Labeling. Ap_4A analogs with modifications to the purine bases or with thiophosphate or methylene, ethylene, or halomethylene phosphonate substitutions in the polyphosphate chain have been extensively studied as activators and inhibitors of various enzymes of Ap_4A metabolism (Blackburn et al., 1992). Some of these compounds can be used to examine the physiological roles of Ap_4A. Here, we have used thiophosphate, methylene and methylene halide substitutions at the ββ′ (i.e., P^2P^3), αβ and α′β′ (i.e., P^1P^2, P^3P^4) positions of Ap_4A as competitors of photolabeling (Figure 7). To compare the effect of various analogs of Ap_4A on photolabeling, we used them at a concentration (4 μM) at which Ap_4A gives 50% reduction in photolabeling (Figure 3D). On the basis of their extent of inhibition of photolabeling, the analogs could be classified into three groups. The first group, with CH_2, CF_2, CCl_2, CHCl or CHF, substitutions in the ββ′ position, was similar

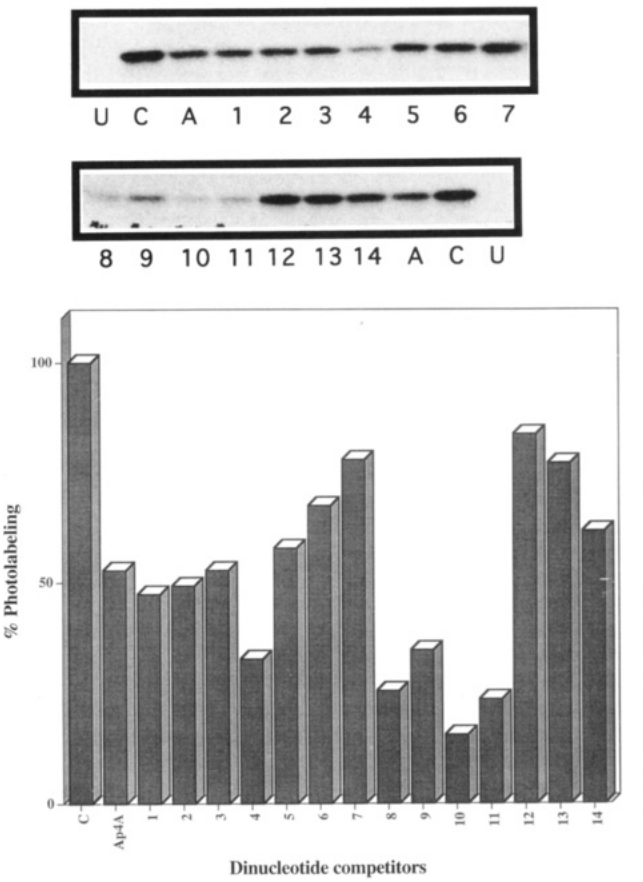

FIGURE 7: Effect of various Ap_4A analogues on photoaffinity labeling. Photoaffinity labeling experiments were set up, and 4 μM Ap_4A or its various analogues were added to the reaction. Upper panels show autoradiograms of one experiment. Lane U represents the result of no UV-labeling. Lane C represents the control lane with no added dinucleoside polyphosphate. Lane A (top panel) or Ap_4A (bottom panel) is with the addition of unmodified Ap_4A. Various Ap_4A analogues are indicated as follows: $AppCH_2ppA$ (lane 1), AppCHFppA (lane 2), $AppCF_2ppA$ (lane 3), AppCHClppA (lane 4), $AppCCl_2ppA$ (lane 5), $AppCH_2CH_2ppA$ (lane 6), $ApCH_2ppCH_2pA$ (lane 7), ϵAppppA (lane 8), $Ap_spCH_2pp_sA$ (lane 9), Ap_sppp_sA (lane 10), Ap_spppA (lane 11), $ApCH_2CH_2ppCH_2CH_2pA$ (lane 12), $ApCCl_2ppCCl_2pA$ (lane 13), and ApCHClppCHClpA (lane 14). The bottom panel is a quantitative representation of the autoradiographic data. The *x*-axis designations are same as for the upper panels.

to unmodified Ap_4A (Figure 7, lanes A and 1−5); the second group comprised ββ′ -CH_2CH_2 and αβ, α′β′ -CH_2 and CH_2-CH_2 derivatives, as well as the αβ, α′β′-CHCl and CCl_2-substituted analogs, and was less effective than Ap_4A (lanes 6, 7, and 12−14); the third group consisted of the monothio and dithio derivatives, Ap_spppA and Ap_sppp_sA, the dithiomethylene compound, $Ap_spCH_2pp_sA$, and etheno-Ap_4A (ϵAppppA). Members of this last group, especially the sulfur-containing compounds, were more effective competitors of photolabeling than Ap_4A itself (lanes 8−11). Modification of one of the adenine rings, as in the fluorescent etheno-Ap_4A (ϵAppppA, lane 8), had a significant effect on its affinity for the 45-kDa protein.

These results show that isosteric and isopolar substitutions are reasonably well tolerated in the ββ′ position; however, the additional length of an ethylene group reduces the affinity somewhat. Isosteric substitution (i.e., CH_2) in both αβ and α′β′ positions also reduces affinity, and increasing the polarity with chlorine atoms does not improve binding. Binding is enhanced by replacing nonbridging oxygens on

the α and α′-phosphorus atoms (P^1 and P^4) with sulfur. However, all the analogs tested are effective competitors of photolabeling. The resistance of these Ap_4A analogs to hydrolysis (Blackburn et al., 1992) should make them valuable tools for elucidating the physiological role of the interaction between the 45-kDa subunit and Ap_4A.

DISCUSSION

Although Ap_4A occurs both in prokaryotes as well as eukaryotes, its role in biological systems is not well defined. In prokaryotes it probably acts as an "alarmone" when the cell is under stress (Kietzler et al., 1992). In eukaryotes it is proposed to be involved in cell proliferation (Rapaport & Zamecnik, 1976), DNA replication (Weinman-Dorsch et al., 1984; Grummt, 1978), and DNA repair (Baker & Ames, 1988). Besides these, antithrombotic and neurotransmitter-like activities are also associated with Ap_4A (Ogilvie, 1992). Earlier we reported the purification and characterization of Ap_4A binding protein associated with DNA polymerase α in HeLa cells (Vishwanatha & Wei, 1992). This purified protein was found to have two subunits of 22 and 45 kDa. Ligands with photoaffinity labels have been used to study active sites of nucleotide binding proteins (Prescott & McLennan, 1990). We have used photoaffinity-labeled Ap_4A in labeling studies and have found that photolabeled Ap_4A binds specifically to the 45-kDa subunit of the Ap_4A binding protein. Binding is saturated at a ligand concentration of 0.4−0.5 μM in the reaction system. Binding was specific for Ap_4A and excess unlabeled Ap_4A competed the photoaffinity labeling reaction. Divalent cations like Zn^{2+} and Mg^{2+} significantly inhibited the labeling of Ap_4A to the 45-kDa protein. This inhibition could be due to a change in conformational stability of the ligand thereby reducing the binding since bivalent cations have been found to alter the spectral characteristics (Holler et al., 1983). Mononucleotides such as dATP and dGTP do not compete the photolabeling, while ATP is only effective at reducing labeling at very high concentrations. This indicates the specific affinity of the protein for dinucleotides.

Dinucleoside polyphosphates of varying phosphate chain length and modified Ap_4A analogs have been used to study the properties of dinucleotide binding proteins. Substitution of purines by pyrimidines has also been carried out to study the physiological role of these compounds. We used some of these analogs, viz.; Ap_4G, Gp_4G, Ap_2A, Ap_3A, and Ap_5A, in our labeling experiments. Substitution of one adenine with guanine did not affect the ability of this dinucleotide to compete for binding, while substitution of both adenines with guanine resulted in no competition with binding of 8-N_3-Ap_4A to the protein. Ap_2A and Ap_3A did not compete for binding while Ap_5A competed for binding. Thus, diadenosine polyphosphates with more than three phosphates can bind efficiently to the protein, indicating that the number of phosphates between the two adenosines may be important. The minimal requirement is a dinucleotide with at least one adenine ring and four phosphates. The chain length specificity is similar to that displayed by eukaryotic Ap_4A hydrolases (Guranowski & Sillero, 1992) and the specific Ap_4A cell surface receptor (Hilderman et al., 1994).

Analogs of Ap_4A such as methylene bisphosphonates and thiophosphates have been used mainly to study the nature of the active sites of dinucleoside polyphosphate hydrolytic enzymes (McLennan et al., 1989; Guranowski et al., 1987, 1989). Many of these analogs have been found to have therapeutic potential in recent years (Blackburn et al., 1992). We used some of these analogs in our studies and found some significant differences in Ap_4A binding to the 45-kDa protein. In general, it was observed that analogs with thiophosphates were more inhibitory than analogs having substitutions in the $\beta\beta^1$ position or halogen-substituted methylene groups in the $\alpha\beta$ and $\alpha'\beta'$ positions.

In order to determine the physiological role of the Ap_4A binding protein in the processes of DNA replication and repair, we are currently determining the amino acid sequence of the purified 45-kDa subunit. Detailed knowledge of the binding protein will enable us to understand the multiple and interesting roles of Ap_4A in eukaryotic cells.

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