Uracil DNA-Glycosylase/Glyceraldehyde-3-Phosphate Dehydrogenase is an Ap_4A Binding Protein[1]

Mayur D. Baxi and Jamboor K. Vishwanatha*

Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-4525

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ABSTRACT: A 37 kDa protein that binds to diadenosine tetraphosphate (Ap_4A) was purified from human HeLa cells and identified as uracil DNA glycosylase/glyceraldehyde-3-phosphate dehydrogenase (UDG/GAPDH). Utilizing photoaffinity labeling with [α-^{32}P]8N_3-Ap_4A, an Ap_4A binding protein of 37 kDa was identified from HeLa cell nuclear extracts. The 37 kDa protein was purified to homogeneity and subjected to trypsin digestion followed by amino acid sequence analysis. Two peptide sequences were determined and both had complete identity with the amino acid sequence of the 37 kDa polypeptide of UDG/GAPDH. Purified UDG/GAPDH binds to Ap_4A with the same affinity as the HeLa cell nuclear 37 kDa Ap_4A binding protein, and monoclonal antibodies to UDG/GAPDH cross-react with the 37 kDa Ap_4A binding protein. UDG/GAPDH has been previously demonstrated to have numerous nonglycolytic activities. The UDG function is involved in DNA repair by excision of uracil from DNA. GAPDH is a RNA binding protein and binds to tRNA and AU-rich RNA. The AU-rich RNA binding has been implicated in the regulation of AU-rich element dependent mRNA turnover and translation. The identification of UDG/GAPDH as an Ap_4A binding protein may be physiologically relevant to the proposed role of Ap_4A as a regulatory nucleotide in cell growth.

Dinucleoside polyphosphates (Ap_nA's) are cellular metabolites which have been found to have varied physiological responses (reviewed in Baxi and Vishwanatha, 1995). The cellular levels of Ap_nA, particularly Ap_4A, rise significantly as a response to stress conditions, both in prokaryotes and eukaryotes (Bochner et al., 1984; Baker and Jacobson, 1986). Studies in the past few years have shown that in addition to its action as a signal molecule, Ap_4A may be involved in metabolic processes associated with cellular stress such as heat starvation and oxidative stress (Kitzler et al., 1992). Although it is not known whether Ap_4A or Ap_nA in general have a protective role in response to stress conditions, there are other indications to show that Ap_4A might be involved in cell proliferation (Rapaport and Zamecnik, 1978; Weinmann-Dorsch et al., 1984, Weinmann-Dorsch and Grummt, 1985; Morioka and Shimada, 1984, 1985), DNA replication (Grummt et al., 1979; Grummt, 1978a, 1978b; Rapaport et al., 1981a; Zamecnik et al., 1982; Zourgui et al., 1984), DNA repair (Remy, 1992), vasotone regulation, and neurotransmission (Ogilvie, 1992). In spite of several reports on the involvement of Ap_4A in DNA replication and repair, the mechanism of involvement of Ap_4A in these two processes is not clearly understood. Ap_4A is a good acceptor of poly ADP-ribose and the latter has been proposed to be involved in DNA repair processes (Durkacz et al., 1980). Poly ADP-ribosylated Ap_4A inhibits *in vitro* DNA replication and is specific for the DNA template used (Baker et al., 1987). Poly ADP-ribose polymerase is elevated during stress and its possible involvement in DNA replication has been suggested (Tanigawa et al., 1978; Boulikas, 1990). These observations may be explained by the proposal that upon DNA damage, Ap_4A is poly ADP-ribosylated and the poly ADP-ribosylated Ap_4A inhibits DNA replication and permits DNA repair. In order to find the role of Ap_4A in DNA replication/repair, we have isolated and characterized one of the Ap_4A binding proteins from nuclear extracts of HeLa cells. In this communication, we describe that uracil DNA glycosylase (UDG)/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Meyer-Siegler et al., 1991) is an Ap_4A binding protein in HeLa cells.

EXPERIMENTAL PROCEDURES

HeLa S_3 cells were grown and fractionated as described earlier (Vishwanatha et al., 1992). In brief, the cells were grown in Joklik's modification of minimal essential medium supplemented with 5% fetal calf serum. Two liter culture of cells (at approx 10^6 cells/mL) were harvested and washed twice with Hank's balanced salt solution and once with hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM $MgCl_2$). The cells were suspended in hypotonic buffer for 2 h at 4 °C and then Dounce homogenized. The homogenate was centrifuged, and the cytoplasmic and nuclear fractions were separated. The nuclear pellet was rinsed three times with hypotonic buffer, and the nuclei were suspended in 0.4 M phosphate buffer, pH 7.2, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. The extraction of proteins was carried out for 1 h at 4 °C on a rocking platform followed by centrifugation of nuclear extract. This nuclear extract was dialyzed against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10% (v/v) glycerol. Dialyzed nuclear extract was loaded onto a Superdex-75 FPLC column (Pharmacia) preequilibrated with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10% (v/v) glycerol. Proteins were eluted at a flow rate

* To whom correspondence should be addressed at: Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 600 S. 42nd Street, Omaha, NE 68198-4525. Phone: 402-559-6663. Fax: 402-559-6650.

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of 0.5 mL/min at 280 nm and 1.0 mL fractions were collected. An aliquot of each fraction was used in measuring Ap_4A binding activity. Peak fractions were collected and concentrated using Centricon-10 centrifugal concentrators (Amicon, MA) to about 200−300 μL. The concentrated fraction was dialyzed against TDEG buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) and subjected to Superose-12 FPLC gel filtration chromatography. Fractions of 1.0 mL were collected at a rate of 0.5 mL/min, and protein elution was monitored by absorbance at 280 nm. The peak fractions were further analyzed for protein concentration, UDG activity, and the presence of 37 kDa Ap_4A binding protein as described below.

Assay of UDG Activity. UDG activity was assayed as described by Meyer-Siegler et al. (1991), using the polynucleotide substrate poly (dA.[^{3}H]dU). In a 100 μL reaction, the enzyme was incubated with 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 5 mM DTT, 20 μg BSA, and 1 μg of the polynucleotide substrate (specific activity: 15,437 cpm/pmol). Reactions were incubated at 37 °C for 1 h, after which 300 μL of ice-cold ethanol, 60 μL of 2 M NaCl, and 100 μL of heat denatured calf thymus DNA (1 mg/mL) were added sequentially. The mixture was kept at −80 °C for 1 h and then centrifuged in a microfuge at full speed for 10 min. Two hundred microliters of supernatant was carefully removed, and the radioactivity in the supernatant was determined by liquid scintillation spectrometry.

Synthesis of Photoaffinity Probe $8N_3$-Ap_4A. $8N_3$-Ap_4A was synthesized as per the method of Prescott and McLennan (1990). All operations involving photoaffinity label (N_3) were carried out in subdued light. Briefly, the synthesis involves mixing 1 μL of 0.3 M $MgCl_2$, 3 μL of 0.1 M $8N_3$-AMP (Sigma Chemical Co., St. Louis, MO), and 250 μCi (^{32}P)ATP (specific activity 3000 Ci/mmole, ICN Biochemicals, Irvine, CA) and evaporation to dryness in a vacuum concentrator. Subsequently, 5 μL of a 1:1 mixture of 3.4 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 triethylammonium 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. The bound nucleotides were eluted with a 20 mL linear gradient of 0.05 M to 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 at 254 nm. Fractions of 1 mL were collected and from each fraction, 1 μL of aliquot was used to determine radioactivity in a scintillation counter. Fractions containing [α-^{32}P]$8N_3$-Ap_4A were combined and concentrated by vacuum concentration. The residues were dissolved in 100 μL of Milli-Q H_2O. Furthermore, the presence of $8N_3$-Ap_4A was qualitatively characterized by thin layer chromatography using butyric acid:H_2O:NH_4OH (66:33:1) as a solvent system (Selvucci et al., 1992). The purity of the $8N_3$-Ap_4A was routinely checked by thin layer chromatography using mononucleotides and dinucleoside polyphosphates as standard. ATP, Ap_2A, Ap_3A, Ap_4A, and Ap_5A were purchased from Sigma Chem Co. (St. Louis, MO).

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, 1 mM ATP, 15 μM $8N_3$-Ap_4A, and indicated amounts of [α-^{32}P]-$8N_3$-Ap_4A. ATP was omitted in experiments to measure the effect of ATP on photolabeling. The reaction mix was incubated in the dark at 37 °C for 1 h, followed by incubation on ice for 5−10 min in an inverted cap of a microfuge tube (in the form of a thin 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 control 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% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 0.01% bromophenol blue). After incubation at 95 °C for 5 min, the samples were loaded onto a 10% denaturing polyacrylamide gel. Electrophoresis was carried out at 150 volts 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 Biosystems Inc., Foster City, CA) of the protein was carried out as per manufacturer's instruction. The Superdex-75 FPLC peak fraction containing 37 kDa protein was photolabeled with [α-^{32}P]$8N_3$-Ap_4A and quenched with SDS buffer as described above. The quenched sample was kept at 95 °C for 5 min. The sample was then loaded onto an 8% polyacrylamide gel (2.5 cm × 100 cm). A linear current gradient of 0.4 mA to 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 protein from the gel was done with 0.25 M Tris-HCl, pH 8.3, at 280 nm. Fractions were collected by peak detection above 5% threshold. The fractions were then analyzed for radioactivity by adding a 1 μL aliquot to 5 mL of Ecolume scintillation cocktail (ICN, Costa Mesa, CA). Fractions containing peak radioactivity in the protein eluting region were further concentrated and analyzed for the labeled protein by SDS-PAGE followed by coomassie blue staining.

Amino Acid Sequencing of Protein. The HPEC purified 37 kDa protein was subjected to SDS-PAGE followed by coomassie blue staining. The 37 kDa protein band was excised from the gel. The gel pieces were destained by repeated washing with 10% methanol and 5% acetic acid. In-gel trypsin digestion was carried out according to the method of Rosenfeld et al. (1992). In brief, the method involves washing the gel piece twice with 50% acetonitrile in 200 mM ammonium bicarbonate pH 8.9 at 30 °C followed by removal of acetonitrile and partially drying the gel piece at room temperature for 10 min. The gel was then rehydrated with ammonium bicarbonate containing 0.02% Tween-20. A 2 μL volume of trypsin (250 μg/mL in ammonium bicarbonate, pH 8.9) was added. A 5 μL volume of ammonium bicarbonate, pH 8.9, was added after the trypsin solution was absorbed. The gel piece was then placed in a microfuge tube, and a minimum amount of ammonium bicarbonate buffer was added to immerse gel piece. The digestion was performed for 10 h at 30 °C and was stopped

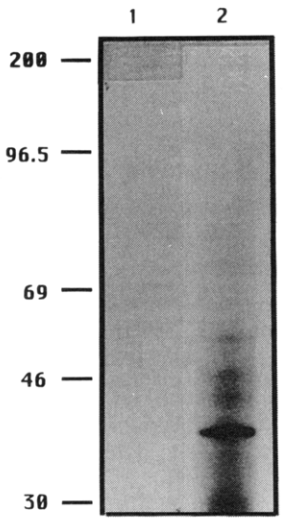

FIGURE 1: Photoaffinity labeling of HeLa cell nuclear extract. HeLa nuclear extract (75 μg of protein) was photoaffinity labeled as described under Experimental Procedures in a reaction containing 3 nM of [α-^{32}P]8N_3-Ap_4A. Labeled proteins were subjected to SDS-PAGE followed by autoradiography. Lane 1 shows ^{14}C molecular weight markers whose sizes are indicated on the *y* axis. Lane 2 represents photoaffinity labeling of the HeLa cell nuclear extract.

by adding 1.5 μL of trifluoroacetic acid (TFA). The digest was collected, and the gel piece was extracted with 100 μL of 60% acetonitrile, 0.1% TFA at 30 °C for 20 min. The extracts were pooled and concentrated to about 20−40 μL by vacuum concentration. The tryptic peptides were separated by reverse phase HPLC. Individual peptides were sequenced by automated Edman degradation using an Applied Biosystems Model 477A. The sequences of the peptides were compared for homology to known amino acid sequences on the PIR protein sequence database.

Protein Estimation. Protein estimations were carried out using Bicinchoninic acid (BCA) method according to manufacturer's instruction (Pierce, Rockford, IL).

RESULTS

Photoaffinity Labeling of a Nuclear Ap_4A Binding Protein. Photoaffinity labeled compounds have been used with success to identify ligand binding proteins. We synthesized photoaffinity labeled Ap_4A, in which one-half of the molecule is tagged with azido group (N_3) and the other half tagged with ^{32}P (Prescott and Mclennan,1990). Due to the high specific activity of the ligand it is advantageous to study the protein−ligand interaction even at lower molar quantities. This ligand, [α-^{32}P]8N_3-Ap_4A, was used in our experiments to photoaffinity label an Ap_4A binding protein from nuclear extracts of HeLa cells. Figure 1 shows the presence of a protein of approximately 37 kDa that is labeled by the photoaffinity analog. This protein band was not visible when the sample was unexposed to UV, indicating that photolabeling is UV dependent. Moreover, the presence of radioactivity in the band suggests that it is the intact Ap_4A molecule and not its degraded product that is cross-linked to the protein. The excess of ATP in the reaction mix (1 mM) also ensures that any nonspecific interaction by adenine moiety is reduced. In order to characterize the photoaffinity labeled nuclear protein, we were interested in studying the amount of photolabel required to saturate the protein. This was essential in order to carry out the ligand binding studies.

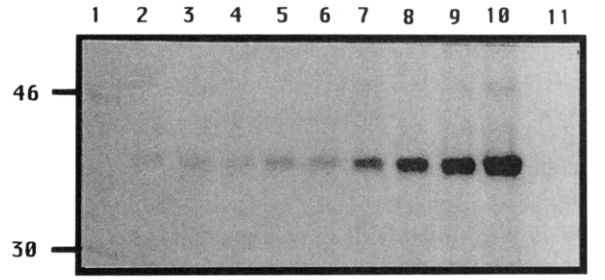

FIGURE 2: Effect of varying concentrations of protein and [α-^{32}P]-8N_3-Ap_4A on photolabeling of 37 kDa protein. The reaction mixture contained 15 μM of 8N_3-Ap_4A and varying concentrations of [α-^{32}P]8N_3-Ap_4A or protein. After photolysis, the reaction mix was electrophoresed on SDS-PAGE and autoradiographed. Lane 1 denotes the ^{14}C molecular weight markers whose sizes are indicated on the *y* axis. Lanes 2−5 show increasing protein concentrations of 3.59 μg, 7.18 μg, 10.77 μg, and 14.36 μg, respectively, incubated in a reaction with 0.45 nM [α-^{32}P]8N_3-Ap_4A. Lanes 6−10 represent increasing ligand concentrations from 0.76 nM to 4 nM, incubated in a reaction with 7 μg of the binding protein.

Increasing concentrations of ligand ranging from 0.76 to 4 nM were used in a labeling experiment. Figure 2 (lanes 6−10) shows that with increasing concentration of radioligand, there was an increase in the intensity of the photolabeled band. At a concentration of about 3.8 nM, no further increase in the intensity of the band was observed as evidenced by the radioactive counts (data not shown). Also, due to the presence of nonradioactive ligand (8N_3-Ap_4A) in the reaction mixture at a concentration 1000-fold more than the labeled ligand, any nonspecific labeling of protein by radioactive ligand is less likely. Besides this, we also noted that with increasing concentration of protein, there was an increase in the intensity of the photolabeled band (Figure 2, lanes 2−5).

Purification and Characterization of the Ap_4A Binding Protein. The 37 kDa protein was purified from the nuclear extract on a Superdex-75 FPLC column. The peak fractions were pooled and concentrated. Upon SDS-PAGE analysis, a major 37 kDa protein band and 3−4 faintly visible protein bands were observed. Upon photoaffinity labeling the pooled fraction, only the 37 kDa protein band was labeled (data not shown). Fractions corresponding to the 37 kDa photolabeled band were used for further characterization of the protein such as requirement for metal ions, competition with nucleotide analogs, and amino acid sequencing. Metal ions such as Mg^{2+} are known to play an important role in the binding of adenine nucleotide to proteins. Moreover, Mg^{2+} may also affect the stability and conformation of the Ap_4A molecule (Holler et al., 1983), and divalent metal ions in general have an effect on Ap_4A binding (Holler, 1992). Figure 3 (panel A) shows the effect of Mg^{2+} on photolabeling of the protein. It was found that in presence of 3 mM Mg^{2+} the photolabeling of protein was more than in the absence of Mg^{2+}. At a near saturating ligand concentration of about 3 nM, the photolabeling in the presence of Mg^{2+} was nearly twice the photolabeling in the absence of Mg^{2+}. This result shows that magnesium enhances the binding of substrate to the protein. It is possible that the presence of Mg^{2+} may stabilize the stacking of two adenine rings of Ap_4A resulting in proper binding. Mg^{2+} has been shown to be required for Ap_4A to bind a receptor protein (Walker *et al.*, 1993). Thus, our results with Mg^{2+} appear to be in agreement with the above observation. In all our following experiments we used

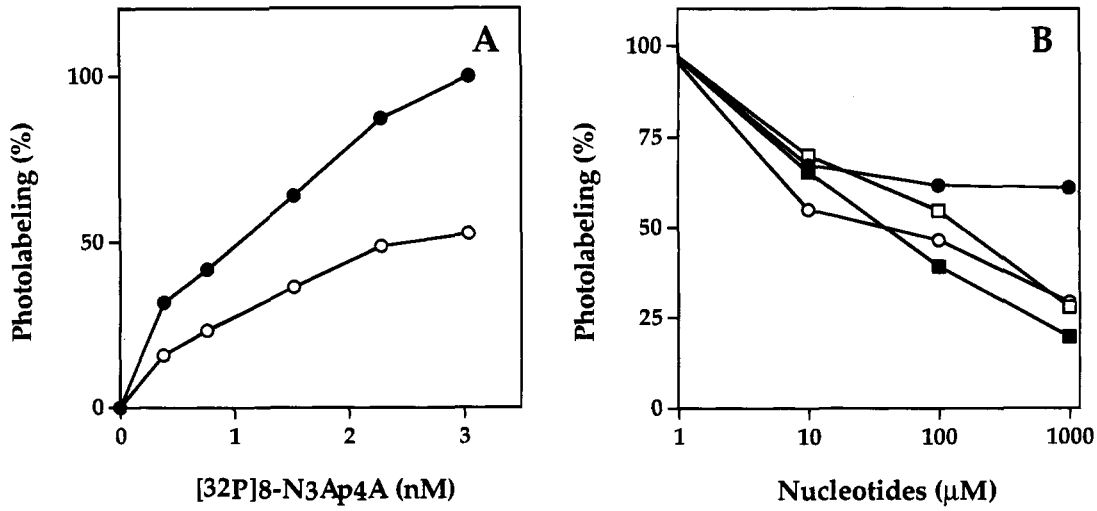

FIGURE 3: Photoaffinity labeling of the 37 kDa protein. A. Photolabeling of 37 kDa protein with varying concentration of [α-^{32}P]8N_3-Ap_4A in the presence and absence of Mg^{2+}. Increasing concentrations of [α-^{32}P]8N_3-Ap_4A from 0 to 3 nM was photolabeled in presence (closed circles) and absence (open circles) of 3 mM Mg^{2+} followed by SDS-PAGE. Each band was measured for radioactivity on a Betascope 603 scanner, and the radioactive counts are represented as % of photolabel incorporated. Each value represents the mean (SE 0−8%) of at least two experiments and triplicate measurements in each experiment. B. Effect of adenine nucleotides on photoaffinity labeling. Purified protein (10.7 μg) was incubated with 4 nM photoaffinity ligand in the presence of varying concentrations of ATP (closed circles), Ap_2A (open squares), Ap_3A (open circles), and Ap_5A (closed squares). Photoaffinity labeling was performed as described in Experimental Procedures. Each value represents the mean (SE 0−10%) of at least two experiments and triplicate measurements in each experiment.

the above two parameters, i.e., presence of Mg^{2+} and appropriate concentration of radioligand (approx 4 nM) in the photolabeling reaction mixture. One way of studying ligand specificity is to use compounds which are structurally similar to that of the parent compound. In characterizing the Ap_4A binding protein, it is important to study the specificity and affinity of Ap_4A to the binding protein. Thus we were interested to see whether other dinucleoside polyphosphates structurally similar to Ap_4A would affect the binding of azido-Ap_4A to the nuclear Ap_4A binding protein. In our experiments we chose ATP (which has a close resemblance to Ap_4A in structure), Ap_2A, Ap_3A, and Ap_5A (which have varying phosphate lengths). Using the ATP and the dinucleotides at a wide range of concentrations, we noted that ATP at a very high concentration (10^5-fold more than the ligand concentration) reduced the labeling by about 40% (Figure 3, panel B). Thus, ATP has less affinity for the protein than Ap_4A. At the same concentration, the dinucleoside polyphosphates reduced photoaffinity labeling by about 70−75%. With increasing phosphate chain length, the amount of photolabeling was reduced. We noted that at 100 μM concentration, with increasing phosphate length Ap_2A → Ap_3A → Ap_5A, there was about a 10% relative reduction in binding. These results show that dinucleotides compete with Ap_4A for binding to the protein, but at a higher concentration. Further, the difference in inhibition by ATP and dinucleotide polyphosphates suggest that the presence of a second adenine may be more competitive for binding. A similar observation has been made by other workers wherein dinucleoside polyphosphates and other nucleotides have been found to reduce the binding of ^{3}H-Ap_4A to DNA polymerase α by 30−40% (Grummt et al., 1979). These observations suggest that the 37 kDa protein has more affinity for Ap_4A than other dinucleotides and ATP.

Analysis of Tryptic Peptides. To identify the 37 kDa Ap_4A binding protein, we carried out amino acid sequence analysis of the protein. Two tryptic peptides of the 37 kDa protein were subjected to amino acid sequence analysis. The resulting sequences were compared for their homology to other known protein in a search of the PIR protein sequence database using the GCG program. We found that both tryptic peptides had a 100% homology with the amino acid sequence of the 37 kDa polypeptide of GAPDH/UDG. Peptide 1 (LVINGNPITIFQER) was identical to amino acid residues 67−80, and peptide 2 (IISNASCTTNCLAPLAK) was identical to amino acids 146−162 of the UDG/GAPDH. To confirm the identity of the Ap_4A binding protein as UDG/GAPDH, we compared commercially available GAPDH, HeLa nuclear extract and purified 37 kDa Ap_4A binding protein using SDS-PAGE and photoaffinity labeling. Commercial GAPDH had the same electrophoretic mobility as the purified 37 kDa Ap_4A binding protein on SDS-PAGE followed by coomassie blue staining (Figure 4, upper left) or after photoaffinity labeling and SDS-PAGE (Figure 4, upper right). This identification was further supported when we observed that a monoclonal antibody (37.04.12) to human UDG/GAPDH cross-reacted with the HeLa 37 kDa Ap_4A binding subunit and the 37 kDa GAPDH/UDG (Figure 4, lower). It has been proposed that human nuclear UDG is the 37 kDa subunit of GAPDH (Meyer-Siegler et al., 1991). This enzyme specifically removes uracil from DNA. Therefore, we were interested in determining the UDG activity of the Hela 37 kDa Ap_4A binding protein. Table 1 shows the UDG activity of the HeLa Ap_4A binding protein in comparison with the activity of the commercially available *E. coli* UDG. On the basis of our results, we conclude that the purified Ap_4A binding protein from the nuclear extract is the 37 kDa subunit of UDG/GAPDH and has the UDG enzymatic activity. To further support our observations that UDG/GAPDH is the 37 kDa Ap_4A binding protein, we used unmodified Ap_4A, Gp_4G, and ATP in separate experiments to see if Ap_4A or Gp_4G compete with photolabeling of the commercial UDG/GAPDH. We used a wide range of concentrations of these nucleotides in the labeling experiment. Figure 5 shows that Gp_4G did not show any significant inhibition except at a very high concentration (2 mM).

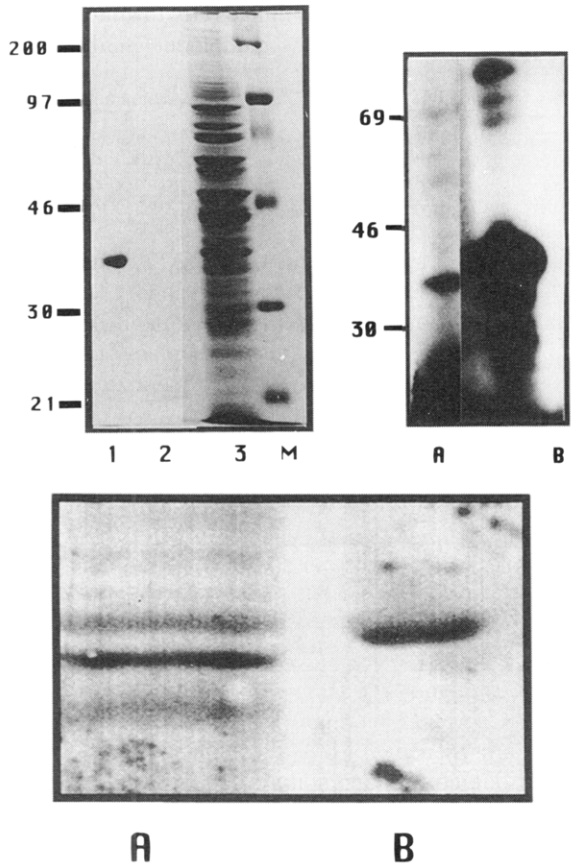

FIGURE 4: The 37 kDa subunit of UDG/GAPDH binds Ap_4A. Upper left: UDG/GAPDH, HeLa cell nuclear extract and purified 37 kDa Ap_4A binding protein were subjected to SDS-PAGE, and the gel was stained with coomassie blue. Lane 1 shows GAPDH, lane 2 shows 37 kDa nuclear protein, and lane 3 shows the HeLa nuclear extract. Lane M shows ^{14}C molecular weight markers whose sizes are indicated on the *y* axis. Upper right: Photoaffinity labeling of GAPDH and purified 37 kDa HeLa cell Ap_4A binding protein. Photoaffinity labeling was performed as described under Experimental Procedures. Lane A shows photoaffinity labeling of the purified HeLa protein and lane B shows labeling of the 37 kDa subunit of GAPDH. Lower: Immunoblot analysis of UDG/GAPDH and HeLa 37 kDa Ap_4A binding protein. Purified HeLa 37 kDa protein (35 μg) and UDG/GAPDH (2 μg protein) were subjected to SDS-PAGE followed by immunoblotting as described previously (Jindal et al., 1991). Mouse monoclonal anti-UDG (37.04.12) primary antibody (3 μg/mL) and alkaline phosphatase conjugated goat anti-mouse secondary antibody were used in immunoblotting. The blots were developed with nitroblue tetrazolium (NBT) and 5-bromo 4-chloro 3-indolyl phosphate (BCIP). Lane A shows the HeLa 37 kDa protein and lane B shows the human UDG/GAPDH.

Table 1: UDG Activity of the HeLa Ap_4A Binding Protein.[a]

protein	UDG activity (units)
E. coli UDG	0.565
HeLa Ap_4A binding protein	0.229

[a] The *E. coli* UDG (0.25 unit) and HeLa Ap_4A binding protein (1.5 μg of Superose-12 purified protein) were assayed for UDG activity as described in Experimental Procedures. One unit of enzyme activity is defined as one picomole of radioactivity released per hour at 37 °C.

Increasing concentrations of Gp_4G (up to 0.02 μM), however, showed an increase in the labeling of a ligand (approximately 2.5 times). We do not know the reason for this increase. In contrast, both ATP and Ap_4A showed almost a linear decrease in photolabeling. In comparison to Ap_4A, the percentage inhibition by ATP was 10−20% lower. This may be due to lower affinity of ATP relative to Ap_4A for the

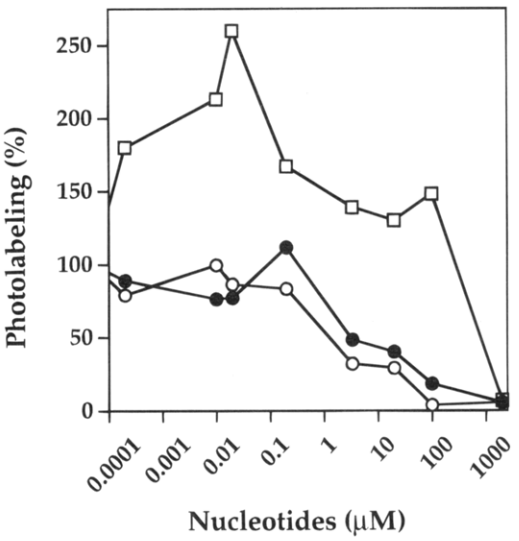

FIGURE 5: Effect of adenine nucleotides on photoaffinity labeling of GAPDH. Four micrograms of commercial GAPDH (tetrameric form) was labeled with 3 nM [α-^{32}P]$8N_3$-Ap_4A in the presence of varying concentrations of ATP (closed circles), Ap_4A (open circles), and Gp_4G (open squares). Following SDS-PAGE, the gel was dried and radioactivity was quantitated with a Betascope 603 radioanalytical imager. Each value represents the mean (SE 0−10%) of at least two experiments and triplicate measurements in each experiment.

binding protein. These results show that Ap_4A does compete with photoaffinity ligand for binding. With respect to specificity of Ap_4A for the binding protein it must be noted that relative inhibition by Ap_4A is more than that of ATP, which indicates that the protein has specificity to Ap_4A. Our observations show that Ap_4A binds to the 37 kDa subunit of UDG/GAPDH. This subunit is also proposed to be involved in DNA repair. Ap_4A has been proposed to be involved in DNA repair although its role in DNA repair is not well established (Remy, 1992). In the light of the above observation, it is possible that Ap_4A may be modulating the activity of UDG.

DISCUSSION

The physiological role of diadenosine tetraphosphate in biological processes is still undefined despite several reports on its involvement in DNA replication/repair, cellular stress, vasotone regulation, and neurotransmission (reviewed in Baxi and Vishwanatha, 1995). Ap_4A interacts with several enzymes and acts as an inhibitor and a transition state analogue of adenine nucleotides such as ATP (Purich and Fromm, 1972; Leinehardt and Secemski, 1973; Bone et al., 1986). It has been used to probe and study several proteins of interest (Sillero and Cameselle, 1992). To understand the role of Ap_4A in DNA replication, several workers have studied the interaction of Ap_4A with DNA polymerase α (Grummt, 1978a, 1978b; Grummt et al., 1979; Rapaport et al., 1981a; 1981b) and have identified and isolated Ap_4A binding proteins from various cells and tissues (Baril et al., 1983; Zourguie et al., 1988; Vishwanatha et al., 1986; Vishwanatha and Wei, 1992; Baxi et al., 1994). To date, however, the mechanism of action of this compound has not been elucidated. Since DNA replication and repair are closely connected processes, many investigators have suggested that Ap_4A and/or its ADP-ribosylated derivatives act as a lever between DNA replication and repair (Baker et al.,

1987; Orfanoudakis et al., 1990; Surowy and Berger, 1983, 1985). In an attempt to study the involvement of Ap_4A in DNA repair/replication we were interested in isolating a protein from the nuclear extracts of HeLa cells. Previous studies on Ap_4A binding proteins have utilized a ^{3}H-Ap_4A binding assay involving charcoal adsorption method. A more accurate and recent approach is the use of photoaffinity ligand of Ap_4A for binding studies (Prescott and McLennan, 1990; Selvucci et al., 1992, 1993). We used [α-^{32}P]8N_3-Ap_4A analogue of Ap_4A to detect Ap_4A binding protein from the nuclear extracts of HeLa cells and demonstrate a 37 kDa protein photolabeled with the radioligand in the presence of excess ATP. Upon further purification, a single polypeptide of 37 kDa was identified whose photoaffinity labeling was enhanced in the presence of Mg^{2+} indicating a requirement of metal ions for optimum binding. This observation is in agreement with observations by others on the requirement of Mg^{2+} in Ap_4A binding (Prescott and McLennan, 1990; Hildermann et al., 1991). The affinity of the 37 kDa protein to Ap_4A is more than other dinucleotide polyphosphates and ATP. The degree of competition appears to increase with an increase in phosphate chain length (Ap_5A > Ap_3A > Ap_2A > ATP). Amino acid sequence analysis of two tryptic peptides of the 37 kDa protein showed a 100% homology with the 37 kDa polypeptide of GAPDH/UDG in the PIR database. The identity of the 37 kDa subunit of GAPDH as Ap_4A binding protein is evident from the photoaffinity labeling of GAPDH with azido Ap_4A and the presence of an immunoreactive band at 37 kDa in HeLa nuclear extracts that also is photolabeled. In competition experiments using ATP, Gp_4G, and Ap_4A, we found that ATP and Ap_4A competed with photolabeling of the UDG/GAPDH, while Gp_4G did not show any significant competition except at a very high concentration. This observation indicates that UDG/GAPDH has more affinity for Ap_4A. GAPDH (EC 1.2.1.12) is a glycolytic enzyme and consists of four identical subunits of 37 kDa in size. Several nonglycolytic functions have been assigned to this protein (Kawamoto and Caswel, 1986; Ronai, 1993) including transcriptional activity (Morgenegg et al., 1986), binding and nuclear transport of tRNA (Singh and Green, 1993), binding to AU-rich RNA, and regulation of mRNA turnover and translation (Nagy and Rigby, 1995). Genetic studies in yeast have revealed that three unlinked genes, TDH1, TDH2, and TDH3, encode GAPDH (Boucherie et al., 1995). The gene product of TDH1 is only synthesized in stressed cells such as in glucose-starvation and heat-shock. Boucherie et al. (1995) proposed that the TDH1-encoded GAPDH plays a cellular role distinct from glycolysis that is required by stressed cells. These observations are significant since Ap_4A levels are increased in cells as a response to cellular stress. The elevated levels of Ap_4A in response to stress has led investigators to term Ap_4A as an "Alarmone" (Lee et al., 1983a, 1983b; Bochner et al., 1986, Varshavsky, 1988) and suggest involvement of Ap_4A in metabolic processes associated in response to stress such as heat shock (Kitlzer et al., 1992). The 37 kDa subunit of GAPDH is also an enzyme that cleaves uracil from DNA (Meyer-Siegler et al., 1991), and this enzyme is involved in DNA repair (Gupta and Sirover, 1981; Domena and Mosbaugh, 1985; Seal et al., 1987). Our experiments suggest that the 37 kDa protein which can be photoaffinity labeled has UDG activity indicating that human UDG is an Ap_4A binding protein. Ap_4A was first shown to be biologically synthesized during protein synthesis in a reaction involving ATP and lysine catalyzed by lysyl tRNA-synthetase (Zamecnik et al., 1966; Randerath et al., 1966; Zamecnik and Stephenson, 1969) and due to this link with protein synthesis was originally proposed to be involved in regulation of translation. Subsequent work on Ap_4A led to the proposal that this compound is involved in DNA replication and repair (Grummt et al., 1979; Baril et al., 1983; Baker and Ames, 1988). In view of these proposed activities of Ap_4A, the identification that UDG/GAPDH is an Ap_4A binding protein may be helpful in eludicating the physiological role of this interesting dinucleotide. In future studies, it would be interesting to examine whether Ap_4A can modulate the activity of UDG in DNA repair in association with DNA polymerase α (Seal and Sirover, 1986). Also, due to the ability of GAPDH to regulate nuclear transport of tRNA (Singh and Green, 1993) and to selectively bind AU-rich RNA in regulating mRNA turnover and translation, future studies on the role of Ap_4A in these processes will establish Ap_4A as a regulatory nucleotide in cell growth.

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