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Adenovirus IVa2 Protein Binds ATP[▽]

Philomena Ostapchuk and Patrick Hearing*

Department of Molecular Genetics and Microbiology, School of Medicine, Stony Brook University, Stony Brook, New York 11794

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IVa2 is an essential, multifunctional protein of adenovirus (Ad) supporting packaging of the viral genome into the capsid, assisting in assembly of the capsid, and activating Ad late transcription. A comparison of IVa2 protein sequences from different species of *Adenoviridae* shows conserved motifs associated with binding and hydrolysis of ATP (Walker A and B motifs). ATPases are essential proteins of bacteriophage packaging motors, and such activity may be required for Ad packaging. Results presented here show that the Ad2 IVa2 protein binds ATP in vitro and that sequences in the Walker A and B motifs are necessary for this activity.

Adenovirus (Ad) IVa2 is an essential, multifunctional protein supporting the packaging of the viral double-stranded DNA genome into the capsid, having an unknown but important role in capsid assembly, and activating Ad transcription at the major late promoter (24, 26, 33, 37–39). Packaging and transcriptional roles of IVa2 require DNA binding, and similar DNA binding motifs are found in the packaging domain at the left end of the viral genome and at the major late promoter downstream elements (17, 29, 37). A mutant virus lacking IVa2 synthesizes late gene products, albeit at reduced amounts compared to those of the wild-type virus; however, the major block for growth of this mutant is in virus assembly (38). Not only does IVa2 interact with the Ad genomes but it is also found in similar, low amounts in empty capsids devoid of viral DNA and in mature virus capsids containing Ad DNA (35, 39).

Alignments of the human Ad2 IVa2 protein sequence along with its predicted secondary structure with IVa2 sequences from representative species of the four genera of *Adenoviridae*, utilizing the ClustalW program and Predator program (7) at the NPS@ Web server (4), showed conservation of amino acid sequence motifs associated with binding and hydrolysis of ATP (19). With ATPases, the Walker A (P loop) and B motifs are involved in binding the ATP-Mg²⁺ ion substrate and are found juxtaposed with beta sheet structures (19, 36). The same is predicted for Ad IVa2 proteins (Fig. 1). In addition, the five highly conserved beta sheets predicted between Ad2 IVa2 amino acids 158 and 426, and the equivalent regions of other Ad IVa2 proteins (Fig. 1), are consistent with the structures of additional strand catalytic E ATPases (16). ATPases are essential proteins for bacteriophage and herpesvirus packaging motors (reviewed in references 2, 9, and 18). Ad assembly intermediates have been identified that suggest that packaging occurs in a fashion similar to that of the DNA bacteriophage, and hence Ad packaging would require an ATPase (5, 6, 13,

32). Likewise, the role of IVa2 in virus assembly and transcription could require ATPase activity. We set out to purify IVa2 to examine if the protein has ATPase activity. Although we were unable to detect ATPase activity associated with purified IVa2 protein, we were able to show that the protein binds ATP and that the binding requires critical residues of the Walker A and B motifs.

Recombinant IVa2 protein containing a C-terminal, eight-amino-acid Strep tag (termed IVa2-Strep) (30) was expressed using a baculovirus vector, following infection of Sf9 cells according to instructions provided in the Bac-N-Bac transfection and expression guide (Invitrogen). The possibility that placing a tag on the C terminus of IVa2 could be detrimental was ruled out by determining that the IVa2-Strep protein was functional. Plasmid pTG3602 (Transgene S.A., Strasbourg, France) (3) was used to derive an IVa2 mutant viral genome, pTG3602ΔIVa2, by deleting Ad5 nucleotides 1343 to 5186; a bovine growth hormone polyadenylation signal was introduced at the site of the deletion to restore a poly(A) signal for E2b mRNAs. The resulting mutant carries deletions of the genes for E1A, E1B, pIX, and IVa2 and does not express a functional IVa2 protein. pTG3602ΔIVa2 was cotransfected with an empty pcDNA3 expression vector (Invitrogen) or pcDNA3 vectors that contained coding sequences for wild-type Ad2 IVa2 or IVa2-Strep. Transfections were performed using FuGENE 6 transfection reagent (Roche) according to the manufacturer's recommended protocol. N52 cells were used to complement the loss of the E1 genes in pTG3602ΔIVa2; the pIX gene is not essential in the context of pTG3602ΔIVa2 since the genome has a reduced size (28). Complementation was measured by overlaying cells 18 h after transfection with Dulbecco's modified Eagle's medium containing 1% agarose and counting plaques that formed 7 to 10 days later (Table 1). The extended deletion in pTG3602ΔIVa2 ensures that virus rescue is due to complementation in *trans* and not due to recombination with IVa2 sequences in the expression vectors. Cotransfection of pTG3602ΔIVa2 with pcDNA3 containing the wild-type IVa2 cDNA (pcDNA3-IVa2-WT) resulted in a substantial number of plaques, demonstrating that the protein was able to rescue the growth of the defective virus. Likewise, cotransfection with

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, School of Medicine, Stony Brook University, Stony Brook, NY 11794. Phone: (631) 632-8813. Fax: (631) 632-8891. E-mail: phearing@ms.cc.sunysb.edu.

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FIG. 1. Ad IVa2 sequence alignments and predicted folding. Results of ClustalW amino acid sequence alignments of human Ad 2 IVa2 with sequences of the indicated Ad species are shown. Protein database locus numbers are as follows: 1, 56160514; 2, 56160919; 3, 2935215; 4, 56158883; 5, 13242719; 6, 56160741; 7, 78068044; 8, 3769487; and 9, 9635798. Mast-, At-, Avi-, and Si- are abbreviations for the following four genera, respectively: *Mastadenovirus*, *Atadenovirus*, *Aviadenovirus*, and *Siadenovirus*. Amino acids 159 to 425 of human Ad2 IVa2 and the equivalent regions of the other Ads are shown. Arrows indicate the predicted beta sheet structures, the dashed line indicates the A box motif (designated A), and the single line indicates the B box motif (designated B). Symbols: identity (*), highly similar amino acids (:), and weakly similar (.) amino acids.

TABLE 1. Transcomplementation of an IVa2 mutant virus by IVa2-Strep proteins

Expression plasmid	No. of plaques ^a		
	Expt 1	Expt 2	Expt 3
pcDNA3	0, 0, 0	0, 0, 0	0, 0, 0
pIVa2-WT	132, 155, 158	73, 98, 115	37, 51, 59
pIVa2-Strep-WT	77, 72, 73	ND	25, 31, 31
pIVa2-Strep-A	ND	0, 0, 0	0, 0, 0
pIVa2-Strep-B	ND	0, 0, 0	0, 0, 0

^a ND, not determined. The numbers of plaques that formed on three separate tissue culture dishes are given.

a plasmid expressing IVa2-Strep (pcDNA3-IVa2-Strep-WT) also yielded numerous plaques but at an approximately twofold lower level than that of the wild-type IVa2. Thus, the IVa2-Strep protein is functional.

IVA2 proteins containing mutations in either the Walker A or B motifs were also analyzed in this assay (pcDNA3-IVA2-Strep-A and pcDNA3-IVA2-Strep-B, respectively) (Table 1). The mutations introduced into each motif are shown in Fig. 2A. The lysine-to-arginine change in the Walker A motif and the aspartic acid-to-asparagine change in the Walker B motif could disrupt critical bonding of the β and γ phosphates of ATP in conjunction with Mg^{2+} ions in the active site of an

FIG. 2. Recombinant IVa2 protein. (A) The schematic illustrates sequence motifs in IVa2. The gray bar represents the IVa2 amino acid backbone with the N-terminal (N-) and C-terminal (-C) ends indicated. The black bars below the gray bar approximate the locations of amino acids important for DNA binding (21, 22), and the striped box represents the location of a nuclear localization signal. The white boxes labeled A and B represent the Walker A and B motifs, respectively. Amino acids encompassing the A and B motifs are shown, and the point mutations introduced into each motif are shown below the downward arrows. (B) Coomassie blue-stained SDS-polyacrylamide gel with 2.5 μ g of purified protein run in each lane. Lanes: 1, molecular mass markers with indicated sizes in kilodaltons; 2, wild-type IVa2-Strep (WT); 3, A box mutant; 4, B box mutant. (C) Electrophoretic mobility shift assay using purified IVa2 proteins. Lanes 1 to 3, wild-type IVa2-Strep protein (WT); lanes 4 to 6, the Walker A motif mutant protein (A Box); lanes 7 to 9, the Walker B motif mutant protein (B Box). IVa2 proteins were incubated in binding reaction mixtures at the indicated concentrations (mM) with a 32 P-labeled DNA probe containing the A1/A2 packaging repeats of Ad5 (23).

ATPase, resulting in the loss of ATP binding and/or ATPase activity (1, 12, 15, 18, 31). The second mutation in the B motif, glutamic acid to aspartic acid, may alter an essential amino acid for ATP hydrolysis (8). It was shown previously that an Ad

genomic clone that contained the conservative point mutation at lysine 180 to arginine in the A motif of IVa2 was defective for growth (25). Consistent with that result, pTG3602IVa2 was not rescued by the IVa2 Walker A motif mutant. Similarly, the IVa2 Walker B motif mutant also was unable to rescue pTG3602IVa2. Similar levels of wild-type and mutant IVa2 proteins were observed in all cotransfections (data not shown). These results demonstrate the importance of these amino acids in the Walker A and B motifs of IVa2 and implicate IVa2 as an ATP binding protein and a potential ATPase.

IVa2-Strep-WT protein was purified in one step from NP-40 lysates (100 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.5 mM dithiothreitol, 1% NP-40) of Sf9 cells by affinity chromatography with Strep-Tactin resin (Qiagen) according to the manufacturer's protocol. Proteins also were purified from lysates expressing Strep-tagged versions of the A and B motif mutants of IVa2. Figure 2B shows a Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel of IVa2 proteins from peak fractions from eluates of the affinity column. A major species was observed for wild-type and mutant IVa2-Strep proteins (Fig. 2B, lanes 2 to 4); quantification showed these proteins to be >95% pure. These proteins had slightly slower mobilities than IVa2 from Ad5-infected cells (data not shown), consistent with the addition of the tag. Several other minor protein bands were observed. The protein band with a slightly slower mobility than full-length IVa2, as well as the bands below the full-length IVa2, are IVa2 related and react with antibodies to both IVa2 and the Strep tag by Western blot analysis (data not shown).

IVa2 binds to a series of repeated sequences, referred to as A repeats (29), within the packaging domain of Ad5 (34, 37). Wild-type IVa2-Strep and the A and B motif mutant proteins were titrated with a 32 P-labeled probe that includes the A1 and A2 repeats (A1/A2) of the packaging domain, and DNA-protein complexes were analyzed by electrophoretic mobility shift assays (Fig. 2C). Conditions were similar to those used previously (23), except that the concentration of poly(dI-dC) was 1 μ g/ml and EDTA was 1 mM. Wild-type and mutant IVa2 proteins bound the A1/A2 packaging sequences with similar affinities. These results demonstrate that the DNA binding activity of IVa2-Strep is preserved and suggests that the purified proteins are folded properly. Furthermore, the mutations in the A and B motifs do not have any significant effect on DNA binding.

Peak fractions containing wild-type and mutant IVa2-Strep proteins were used in ATPase assays. No significant ATPase activity was observed in assays performed (as described in reference 20) using [α - 32 P]ATP as a substrate and determining hydrolysis by thin-layer chromatography in reactions where the concentrations of protein, ATP, NaCl, or pH were varied or DNA corresponding to the Ad packaging domain was added (data not shown). In addition to examining ATPase activity, wild-type and mutant IVa2 proteins were tested for binding of ATP by UV cross-linking to [α - 32 P]ATP (Fig. 3). These reaction mixtures contained 2.6 μ M protein, 1 μ M [α - 32 P]ATP (specific activity, 3 mCi/nmol), 10 mM Tris (pH 7.4), 5% glycerol, 300 mM NaCl, 1 mM EDTA, and 0.5 mM dithiothreitol. Cross-linking was done on ice for 30 min using a Stratalink (Stratagene) at a dose of $\sim$ 5.4 J/cm². Reactions were terminated by addition of an equal volume of SDS sample buffer,

FIG. 3. The IVa2 protein binds ATP. (A) UV cross-linking experiment with [α - 32 P]ATP and purified, wild-type IVa2-Strep protein either in the absence (–) or presence (+) of MgCl₂, as indicated in lanes 1 and 2. Following cross-linking, samples were analyzed by SDS-polyacrylamide gel electrophoresis and the gel was exposed to a phosphorscreen. (B) Coomassie blue-stained SDS-polyacrylamide gel of UV cross-linked samples. Lane 1, molecular mass markers with indicated sizes in kilodaltons; lanes 2 and 3, wild-type IVa2-Strep protein (WT); lanes 4 and 5, A motif mutant IVa2-Strep protein (A Box); lanes 6 and 7, B motif mutant IVa2-Strep protein (B Box). Lanes 2, 4, and 6 are from reactions done in the presence of MgCl₂, and lanes 3, 5, and 7 are from reactions done in the absence of MgCl₂. (C) Radiolabeled image from the UV cross-linking experiment of wild-type and mutant IVa2-Strep proteins either in the absence (–) or presence (+) of MgCl₂, as described for panel B.

and the samples were electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was fixed, stained with Coomassie blue, dried, and exposed to either X-ray film or a phosphorscreen. 32 P labeling of the wild-type IVa2-Strep protein resulted in a 6-

to 10-fold higher signal in the presence of 5 mM MgCl₂ than in the absence of added MgCl₂ (Fig. 3A). The 32 P-labeled band migrated at the same position on the SDS-polyacrylamide gel as the purified IVa2-Strep protein. Similar results were obtained using protein concentrations between 1 and 6 μ M with 1 μ M ATP (specific activities between 0.9 and 3 mCi/nmol) and with two independent preparations of wild-type IVa2-Strep protein (as well as A and B motif mutant proteins; see below).

Cross-linking of [32 P]ATP to the Walker A and B motif mutant proteins (3.2 μ M in the reaction mixture) was reduced to levels similar to those observed with wild-type IVa2 protein in the absence of MgCl₂ (Fig. 3C). The 32 P-cross-linked proteins shown in Fig. 3C correspond to the IVa2 proteins observed in the Coomassie blue stain of the same gel (Fig. 3B). Similar amounts of protein were recovered from all the cross-linking reaction mixtures (Fig. 3B). These results show that the Ad2 IVa2 protein binds ATP in a manner dependent on the integrity of the Walker A and B motifs.

The absence of significant ATPase activity with the purified IVa2 protein may reflect a low binding affinity for ATP. Alternatively, the IVa2 protein may bind and hydrolyze a different nucleotide triphosphate than ATP. However, in vitro assays using different cold nucleotide triphosphates as competitors in cross-linking reactions with [α - 32 P]ATP and wild-type IVa2 protein did not support this idea (data not shown). Two additional Ad proteins have been shown to be important for packaging of the viral genome, the L4 22-kDa protein and the L1 52/55-kDa protein (10, 14, 23). The L4 22-kDa protein binds to sequences within the packaging domain adjacent to the IVa2 binding sites; the binding of IVa2 in conjunction with the L4 22-kDa protein is essential for packaging (23). The L1 52/55-kDa protein has been shown to associate with the packaging domain in vivo (24, 27) and has been shown to associate with IVa2 in vitro (11). It is possible that ATPase activity of IVa2 requires one or both of these Ad proteins, a possibility that requires further experimentation.

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