

Use of acetone to attain highly active and soluble DNA packaging protein Gp16 of Phi29 for ATPase assay

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Abstract

All the well-defined DNA-packaging motors of the dsDNA viruses contain one pair of nonstructural DNA-packaging enzymes. Studies on the mechanism of virus DNA packaging have been seriously hampered by their insolubility. Phi29's DNA-packaging enzyme, gp16, is also hydrophobic, insoluble, and self-aggregating. This article describes approaches to obtain affinity-purified, soluble, and highly active native gp16 with the aid of polyethylene glycol or acetone. The specific activity of this native gp16 was increased 3400-fold when compared with the traditional method. This unique approach made the ATP–gp16 interaction study feasible. Gp16 binds strongly to ATP, binds to ADP with a lower efficiency, and binds very weakly to AMP. The order of gp16-binding efficiency to the four ribonucleotides is, from high to low, ATP, GTP, CTP, and UTP. The ATP concentration level required to produce 50% of maximum virus yield exhibited during *in vitro* phi29 assembly is around 45 μM , which is close to the gp16 and ATP dissociation constant of 65 μM . Mutation studies revealed that changing only one conserved amino acid, whether R¹⁷, G²⁴, G²⁷, G²⁹, K³⁰, or I³⁹, in the predicted Walker-A ATP motif of gp16 caused ATP hydrolysis and viral assembly to cease, while such mutation did not affect gp16's binding to ATP. However, mutation on amino acids G²⁴⁸ and D²⁵⁶ did not affect the function of gp16 in DNA packaging.

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Introduction

One striking feature in the assembly of linear dsDNA viruses, including bacteriophages, adenoviruses, poxviruses, and herpesviruses, is that the viral genome is packaged into a performed procapsid during maturation (Bazin et al., 1985; Casjens and Hendrix, 1988; Black, 1995; Lee and Guo, 1994). DNA packaging as an essential viral replication step has been investigated in the quest for new antiviral drugs. Factors involved in DNA packaging have been used as model targets for inhibiting viral replication (Trottier et al., 1996; Zhang et al., 1995) and for the development of drugs against CMV (Bogner, 2002). All DNA-packaging motors involve nonstructural components. These components have been classified into two categories according to their role in DNA packaging: procapsid binding (the larger subunit) and interaction with DNA (the smaller sub-

unit) (Guo et al., 1987b). Procapsid binding components include gpA in phage lambda (Catalano et al., 1995), gp12 in phi21 (Feiss et al., 1985), gp17 in T4 (Rao and Black, 1988), gp19 in T3 and T7 (Morita et al., 1993), and gp16 (?) or pRNA in phi29 (Guo et al., 1987a; Garver and Guo, 1997; Shu and Guo, 2002; Guo, 2002). Components interacting with DNA include gpNu1 in lambda, gp1 in phi21, gp16 in T4, gp3 in phi29, and gp18 in T3/T7. Emerging information reveals that the mechanism of herpesvirus DNA packaging is very similar to that of phages (Newcomb et al., 2001; Hwang and Bogner, 2002). Studies on the mechanism of DNA packaging and the design of drugs targeting the DNA-packaging protein have been seriously hampered by the insolubility of this kind of protein after expression and their self-aggregation after purification. Since most of these proteins have been cloned, overexpressed, and purified, some interesting data on the ATPase activity assay of the DNA-packaging proteins have been reported (Hwang et al., 1996; Tomka and Catalano, 1993; Grimes and Anderson, 1990; Guo et al., 1987b; Hamada et al., 1987; Mitchell et al., 2002). However, progress in the stoichiometric quanti-

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fication and mechanistic investigation of these proteins has been far from satisfactory. Not a single crystal structure of these proteins has been reported.

The hydrophobicity, insolubility, and self-aggregation of phi29 gp16 have hindered further refinement of our understanding of the mechanism of this DNA-packaging motor. Contradictory data regarding ATPase activity, binding location, and the stoichiometry of gp16 have been published (Guo et al., 1987b; Grimes and Anderson, 1990; Ibarra et al., 2001). All discrepancies have arisen due to the lack of a method to make gp16 completely soluble. Little progress has been made since the gene coding for gp16 was cloned into *Escherichia coli* and overproduced 15 years ago (Guo et al., 1986). In that traditional method, gp16 was purified in denatured condition and active gp16 was obtained by dialysis against 4 mM KCl buffer for 40 min that allowed for renaturation. Nevertheless, the gp16 aggregated again within 20 min after renaturation. Recently, it was reported that gp16 was made soluble in the cell by coexpression with groE (Ibarra et al., 2001). Indeed, active gp16 has been purified from this overexpression system. However while the coexpression with groE solved the problem of aggregation in the cell, it could not solve the problem of self-aggregation after purification. There is a great need for a procedure to make pure, soluble, active, and stable gp16, which would allow for further characterization of such a fascinating molecular motor.

This article describes approaches utilized to obtain homogeneous, soluble, active, and stable gp16 by the use of a Ni-agarose column, polyethylene glycol (PEG), or acetone. This gp16 construction and preparation enables us to provide the first direct evidence that gp16 specifically binds to ATP. The interactions of gp16 with ATP, ADP, and AMP as well as GTP, CTP, and UTP were also characterized. Nine site-directed mutant gp16s with mutation at the predicted Walker-A motif were also constructed and purified. With the ATPase assay, the results confirmed that the Walker-A motif involves G²⁷I²⁸G²⁹K³⁰S³¹.

Results

PEG and acetone greatly enhance the solubility and activity of gp16

The His-tagged gp16 purified by a Ni-agarose column was completely inactive in phi29 DNA packaging after column elution. The loss of activity was due to self-aggregation of the His-tagged gp16 after purification, since the formation of white precipitates was observed.

The effect of individual organic solvents on gp16 activity was tested. PEG (Huang and Guo, 2003) and acetone were the chemicals most efficient in the restoration of gp16 activity. The affinity-purified gp16 was dissolved in PEG buffer (15% PEG 8000, 5% glycerin, 100 mM NaCl, 20 mM Tris-HCl, pH 7.8) and was active following storage at less

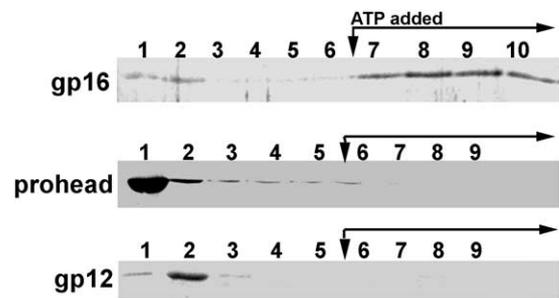


Fig. 1. ATP-binding assay for His-tagged gp16 with ATP-agarose affinity column. His-tagged gp16, procapsid, and gp12 in binding buffer were applied onto an ATP-agarose affinity column and washed with binding buffer. The pass-throughs were collected as fractions. The mark "ATP-added" indicates that elution buffer containing 10 mM ATP was added to the column when fraction 6 or 7 was collected. These fractions were further applied to 10% SDS-PAGE and protein bands were detected by silver stain. The unbound protein appeared in the first several fractions (the void volume). His-tagged gp16 was eluted out with the addition of ATP, while procapsid and gp12 were not.

than -20°C for at least 1 year. Five percent of acetone could replace 15% PEG, but gelatin and Tween 20 could not. This PEG buffer not only could be used for native His-tagged gp16, but also could be used to renature the gp16 that had been denatured by guanidine chloride (GuCl) and to enhance its activity as well. With PEG, the specific activity of native gp16 increased 3400-fold in comparison to the traditional GuCl-KCl method (Huang and Guo, 2003).

Binding of gp16 to ATP

An ATP-affinity agarose column was used to detect whether the native, soluble, and pure His-tagged gp16 could bind ATP. Purified His-tagged gp16 in binding buffer was applied to an ATP affinity column. The majority of the His-tagged gp16 was found to bind to the ATP matrix (Figs. 1-3,5). The bound His-tagged gp16 was eluted by a binding buffer containing ATP. When procapsid, or gp12 (Fig. 1), was applied to the column, it was found that neither of these components bound to the ATP column and that the majority appeared in the first several fractions (the void volume); neither of the proteins were eluted with the addition of ATP. These results provide strong evidence that His-tagged gp16 specifically binds to ATP. Similarly, wild-type gp16 without the fusing tag and prepared by the traditional method (Guo et al., 1986) also specifically bound to the ATP column and was eluted by ATP (data not shown). This suggests that such binding to ATP is an intrinsic feature of gp16, one that is independent of the fusing tag.

Comparison of gp16-binding efficiency to ATP, ADP, and AMP

ATP, ADP, and AMP-affinity agarose resins, attached at the C-8 position to cyanogen bromide-activated agarose, were tested to compare their binding efficiencies to His-

tagged gp16 (Figs. 2A, D, and E). When binding buffer containing ATP was applied to the column, a large amount of His-tagged gp16 was eluted, as detected by *in vitro* assembly assay. The elution profile obtained with the *in vitro* phi29 assembly assay agreed with the data from the dot-blot protein assay, SDS-gel electrophoresis, and silver stain detection. Very little His-tagged gp16 was eluted from AMP-affinity column when it was eluted with binding buffer containing AMP. The ratio of the eluted peak and the peak in void volume was about 10^4 for ATP, 0.7 for ADP, and 0.2 for AMP (Fig. 2) (Table 1).

Binding efficiency of gp16 to ATP with different linkage positions to agarose resin

ATP resins with different attachment positions were used to compare relative differences in His-tagged gp16-binding efficiency. It was found that the ATP resin with an attachment position of C-8 had the highest binding efficiency, while those with an attachment position of N-6 or ribose hydroxyl exhibited a much lower binding efficiency (Figs. 2A, B, and C). Similarly, His-tagged gp16 could not bind to ADP resin with the attachment position of ribose hydroxyl (data not shown).

Comparison of gp16-binding efficiency for ATP, CTP, GTP, and UTP

To compare the binding efficiency for ATP, CTP, GTP, and UTP, His-tagged gp16 was first attached to the ATP-agarose gel. After washing with an excess amount of binding buffer, the bound His-tagged gp16 was then eluted by the buffer containing 10 mM of ATP, CTP, GTP, and UTP, respectively (Fig. 3). It was found that ATP buffer could elute the bound His-tagged gp16 effectively, while the UTP and CTP buffers were less efficient. The binding efficiency of GTP fell between that of ATP and UTP. This indicates that His-tagged gp16 has the highest affinity for ATP binding and the lowest affinity for UTP and CTP.

Determination of dissociation constant (K_d)

The K_d for free ATP and His-tagged gp16 was determined to be 65 μ M (Fig. 4A) using the *double reciprocal plotting* method. This determination is done by a Microcon spin column approach (see Materials and methods). The concentration requirement of ATP for phi29 assembly was determined through titration by the *in vitro* assembly system (Fig. 4B). The minimum ATP concentration required to produce a maximum yield of phi29 was about 100 μ M. The ATP concentration required for half-activity was 45 μ M, which is close to the dissociation constant of 65 μ M.

Mutation on the putative Walker-A motif

Nine site-directed mutant His-tagged gp16s (Table 2) were constructed with mutation at conserved amino acids of predicted Walker-A motif (residues 17–31). This ATP motif has been predicted by sequence comparison (Guo et al., 1987b). ATPase and DNA-packaging assays showed that changing any one of the conserved amino acids, $K^{30} \rightarrow T$, $R^{17} \rightarrow E$, $G^{24} \rightarrow D$, $G^{27} \rightarrow D$, $G^{29} \rightarrow S$, $K^{30} \rightarrow H$, $I^{39} \rightarrow K$, and $I^{39} \rightarrow E$, in the putative Walker-A motif hindered gp16 activities (Fig. 6 and Table 2). ATP-binding assay showed that this loss of activity was not due to a loss of binding of gp16 to ATP (Fig. 5 and Table 2).

A notable phenomenon in mutagenesis study of gp16s was observed: All gp16s with a mutation at the putative Walker-A motif exhibited lower soluble protein yields during protein purification (Table 2). Wild-type gp16 is hydrophobic and tends to self-aggregate. Mutation deteriorates the solubility of gp16; as a result, purification of all of these mutant gp16s with mutations targeting at the putative Walker-A motif proved to be difficult. This was especially true when I_{39} was changed to K or E. Mutant gp16s with $G^{29} \rightarrow S$ & $K^{30} \rightarrow I$, $G^{27} \rightarrow D$, $R^{17} \rightarrow E$, or $G^{24} \rightarrow D$ were purified, taking advantage of the His-tag column's simplicity. These mutant gp16s were found to be able to both bind ATP using S-tag Western blot assay (Fig. 5) and inhibit phi29 assembly. We also tried to study mutations of other gp16 residues. It was found that changing G^{248} to S, or D^{256} to H, did not affect the activity of gp16 (Table 2).

ATPase activity of gp16 and mutants

Thin-layer chromatography revealed that the mixture of procapsid, pRNA, and DNA-gp3 (lanes 2, 10 of Fig. 6) contained very little ATPase activity. With the addition of His-tagged gp16 to this reaction mixture, ATPase activity increased dramatically (lanes 8, 16 of Fig. 6). The purified His-tagged gp16 alone also exhibited very low ATPase activity (lanes 7, 15 of Fig. 6). This result confirmed our previous finding (Guo et al., 1987b) that the ATPase activity of gp16 is procapsid dependent. When mutant His-tagged gp16s, $G^{29} \rightarrow S$ & $K^{30} \rightarrow I$, $G^{27} \rightarrow D$, $R^{17} \rightarrow E$, or $G^{24} \rightarrow D$, were added to the mixture, little ATPase activity was detected (lanes 4, 6, 12, and 14 of Fig. 6). This data indicate that residues R^{17} , G^{29} , K^{30} , G^{27} , and G^{24} are critical for ATPase activity and confirms that $G^{27}I^{28}G^{29}K^{30}S^{31}$ is in the Walker-A motif.

Discussion

Interactions between DNA and cationic polymers, including polylysine and PEG, have attracted attention. PEG has been found to enhance the efficiency of DNA packaging (Serwer et al., 1983; Louie and Serwer, 1994). Cationic polymers appear to have the efficiency to regulate the phys-

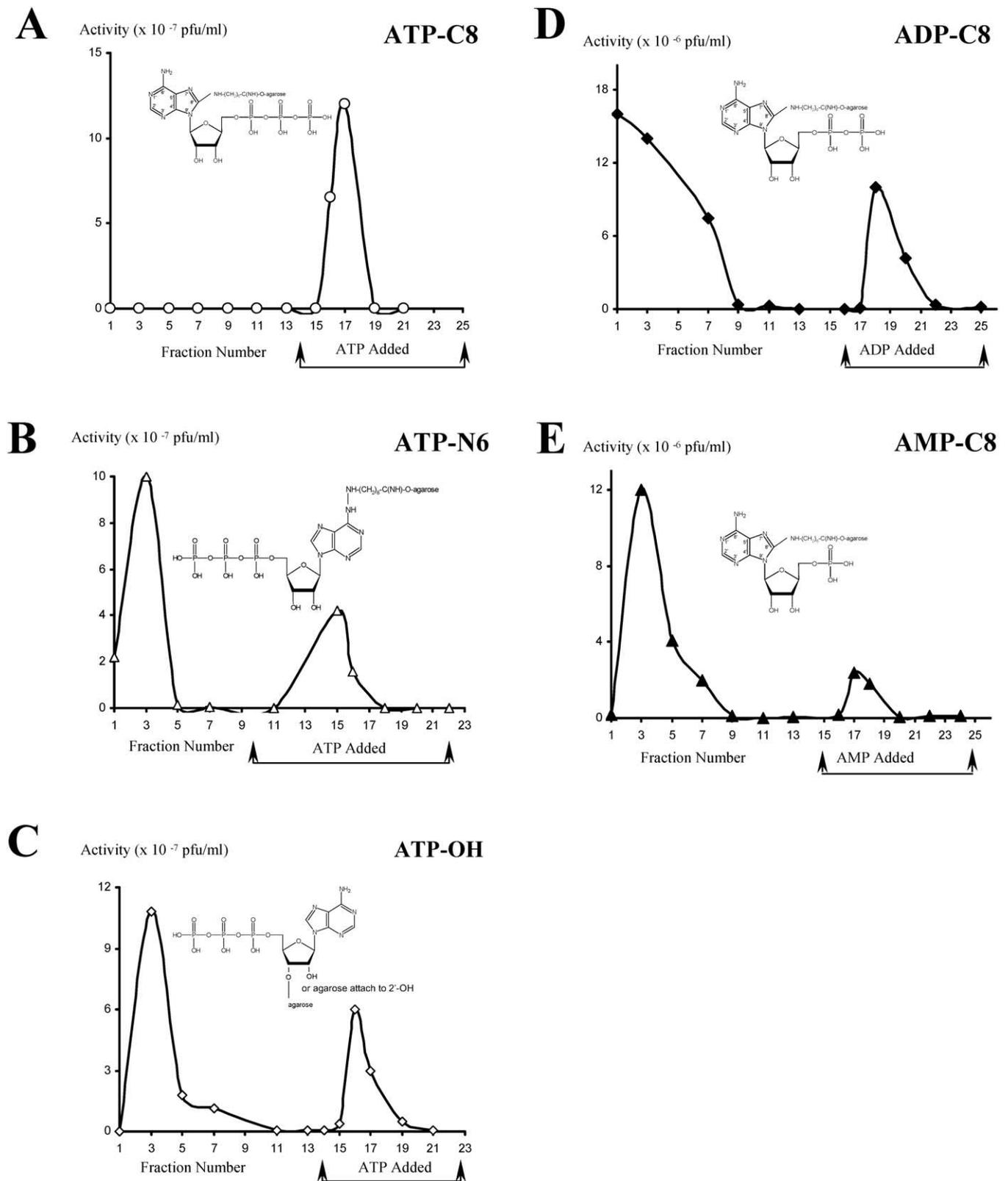


Fig. 2. Comparison of His-tagged gp16-binding efficiency to ATP-agarose resins with different sites of linkage (A–C) and His-tagged gp16-binding efficiency to ATP, ADP, and AMP (A, D, E).

Table 1
Comparison of gp16 binding efficiency for nucleotides

| Nucleotides | Peak activity in void volume (PFU/ml) | Peak activity in elution volume (PFU/ml) | Ratio of peak activity (gp16 ^b /gp16 ^{ub}) | Ranking of Gp16 binding affinity |
|-------------|---------------------------------------|--|---|----------------------------------|
| ATP | 1.1×10^4 | 1.2×10^8 | 1×10^4 | 1 |
| ADP | 1.4×10^7 | 1.0×10^7 | 0.7 | 2 |
| AMP | 1.2×10^7 | 2.4×10^6 | 0.2 | 3 |

Gp16^b, bound gp16; Gp16^{ub}, unbound gp16.

icochemical and biological properties of polycation/DNA complexes. Polymers promote the formation of complexes by facilitating the oriented self-assembly of DNA with cationic-hydrophilic copolymers, which could enshroud the complex within a protective hydrophilic polymer corona. Therefore, polymers could stimulate the packaging of nucleic acid in vitro (Toncheva et al., 1998; Yoshikawa, 1997). The phenomenon of PEG enhancement has been connected to the mechanism of viral DNA packaging (Serwer et al., 1983; Louie and Serwer, 1994), and our results indicate that PEG does indeed enhance the packaging efficiency of phi29. However, it is clear from this study that this enhancement is mainly due to the promotion of gp16 solubility. Whether the aforementioned hydrophilic polymer corona effect could facilitate the packaging of phi29 DNA remains to be investigated.

Other organic solvents such as chloroform, n-octyl- β -D-

glucoside (OG), ethanol, and acetonitrile were found ineffective, likely due to the solvents' attendant inactivation of other protein components involved in the assembly of infectious phi29 virus. This by no means indicates that other organic solvents could not help to dissolve gp16 and restore its activity.

Comparison of the DNA-packaging proteins, UL15 of herpesvirus (Yu and Weller, 1998), IVA2 of adenovirus, A32 of poxvirus (Amegadzie et al., 1991), gp16 of phi29 (Guo et al., 1987b), gp19 of T3 (Yamada et al., 1986), gp17 of T4 (Black, 1995), gp2 of P22 (Eppler et al., 1991), B protein of P1 (Skorupski et al., 1992), terminase of psiM2 (Pfister et al., 1998), terminase of Sfi21 (Desiere et al., 1998), gpA of lambda (Catalano et al., 1995), gp1Y of phiC31 (Bentley, 2002), and gp19 of T7 (Serwer et al., 1983), reveals that most of these proteins contain a conserved Walker-A motif (G/A)XXXXGK(S/T) (Guo et al., 1987b; Mitchell et al., 2002).

We also compared gp16's binding with different agarose

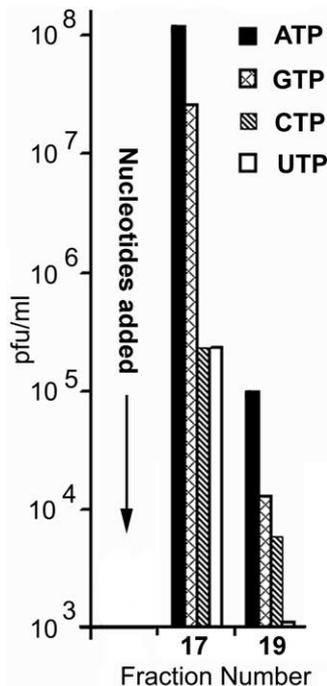


Fig. 3. Comparison of His-tagged gp16-binding efficiency to ATP, GTP, CTP, and UTP. His-tagged gp16 was applied onto an ATP-agarose affinity column and washed with binding buffer. His-tagged gp16 was eluted by 10 mM ATP, GTP, CTP, and UTP. The arrows indicate that the specific nucleotide was added to the binding buffer.

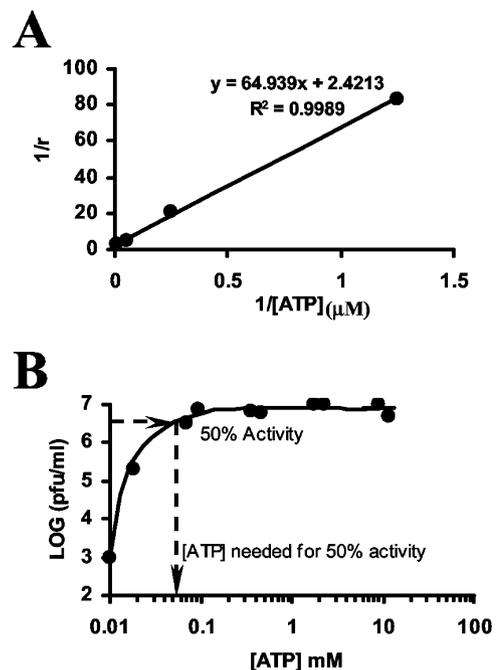


Fig. 4. (A) Double-reciprocal plot to determine the dissociate constant (K_d) of His-tagged gp16/ATP complex. The slope of this line, 65 μ M, stands for the K_d of His-tagged gp16 and ATP. (B) ATP concentration requirement for virion assembly with the standard phi29 in vitro assembly system.

Table 2
Summary of Mutant gp16s

| Location of mutation | Change of residues | Assembling activity (PFU/ml) | Solubility | ATP binding | Procapsid/DNA dependent ATPase activity |
|--------------------------|---|------------------------------|------------|-------------|---|
| Predicted Walker-A motif | G ²⁹ → S & K ³⁰ → I | 0 | ++ | ++++ | – |
| | K ³⁰ → T | 0 | – | N/A | N/A |
| | R ¹⁷ → E | 0 | + | ± | – |
| | G ²⁴ → D | 0 | + | ++ | – |
| | G ²⁷ → D | 0 | ++ | ++++ | – |
| | G ²⁹ → S | 0 | ± | ++ | N/A |
| | K ³⁰ → H | 0 | ± | ++ | N/A |
| | I ³⁹ → K | 0 | – | N/A | N/A |
| | I ³⁹ → K | 0 | – | N/A | N/A |
| Other mutations | G ²⁴⁸ → S | 10 ⁸ | ++ | N/A | N/A |
| | D ²⁵⁶ → H | 10 ⁸ | + | N/A | N/A |
| | Wild-type gp16 | 10 ⁸ | ++ | +++ | ++ |

PFU, plaque forming unit; N/A, data not available; –, not soluble or no ATPase activity.

linkage sites of ATP to see which linkages block the protein/ATP interaction. If a site of ATP is important for gp16 binding, modifying this site by attaching agarose will definitely affect its binding efficiency with gp16. ATP-binding assays showed that ATP resin with an attachment site of C-8 had the highest binding efficiency for gp16, while with an attachment site of N-6 or ribose hydroxyl showed lower binding efficiency (Fig. 2). This suggests that N-6 or ribose hydroxyl is important for ATP/gp16 interaction, a speculation that is further supported by the ADP-binding assay. ADP columns with an attachment site of N-6 or ribose hydroxyl exhibit almost no binding to gp16. Studies on the crystal structure of other ATPases that have a conserved Walker-A motif, such as MinD (Hayashi et al., 2001), have revealed that N-6 and ribose hydroxyls are needed to secure the ATP at binding pockets by forming hydrogen bonds with backbone amino acids. N-6 forms hydrogen bonds with the “O=C—” group of Asn or Pro. Ribose hydroxyl forms hydrogen bonds with the “OH—” group of Ser or Thr, and with the “—NH₂” group of Arg, His, or Lys. The C-8 position did not show specific interaction with backbone amino acids. Since gp16 contains the conserved Walker-A motif, it is not surprising to find that linkage

through N-6 and ribose hydroxyls blocks their interaction with gp16. It is also likely that the poor binding affinities of N-6 and ribose hydroxyl columns are due to a steric effect

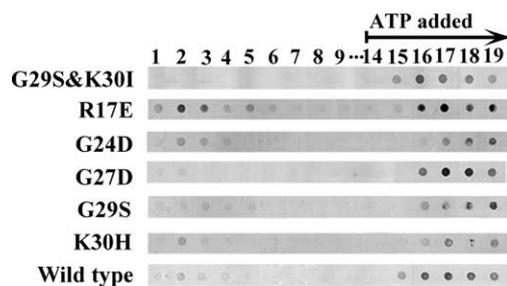


Fig. 5. S-Tag Western blot assay for ATP binding efficiency of mutant His-tagged gp16s. The series of dots represents the fractions as labeled at the top. Data of fractions 10–13 were omitted from this figure.

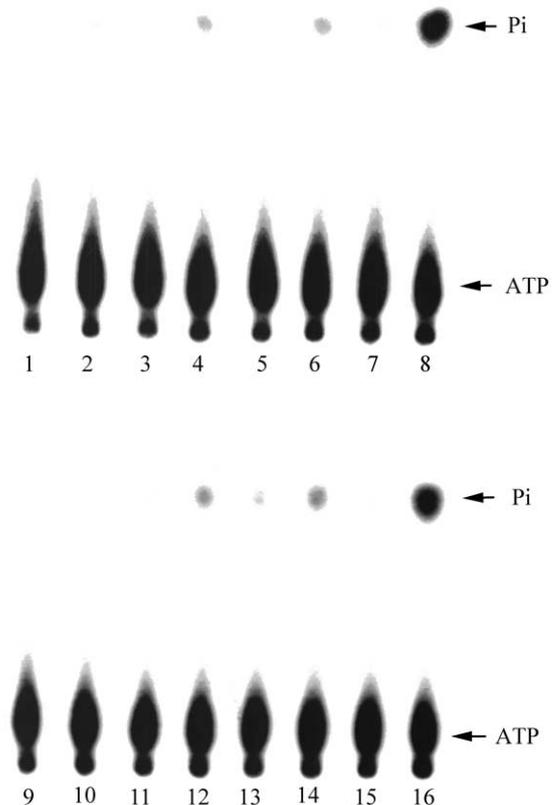


Fig. 6. Thin-layer chromatogram showing hydrolysis of [γ -³²P]ATP by gp16 and related mutants. Lane 1; [γ -³²P]ATP only; 2; proheads, RNA, and DNA-gp3; 3; mutant G29S&K30I; 4; proheads, RNA, DNA-gp3, and mutant G29S&K30I; 5; mutant G27D. Lane 6: proheads, RNA, DNA-gp3, and mutant G27D; 7; His-tagged gp16; 8; proheads, RNA, DNA-gp3, and His-tagged gp16; 9; [γ -³²P]ATP only; 10; proheads, RNA, and DNA-gp3; 11; mutant G29S; 12; proheads, RNA, DNA-gp3, and mutant G29S; 13; mutant K30H; 14; the proheads, RNA, DNA-gp3, and mutant K30H; 15; His-tagged gp16; 16; proheads, RNA, DNA-gp3, and His-tagged gp16.

of altered orientation of nucleotide. This is another area for potential investigation.

Gp16 had higher affinity for ATP than for ADP and AMP. Since the ATP, ADP, or AMP resins used in these assays all link to agarose at the same C-8 position, the resulting steric effects are the same and thus their binding affinities with gp16 are comparable (Figs. 2A, D, and E) (Table 1). Our agarose gel binding assay revealed that gp16 had a high affinity for ATP, while having a lower affinity for ADP and AMP. All of these findings help explain why gp16 and ATP are needed for the packaging of viral DNA. Exhibiting a higher affinity for ATP, gp16 will trap ATP. Having less affinity for ADP and AMP, gp16 will release ADP or AMP after hydrolysis. The alternative binding and releasing of ATP and its hydrolyzed substrate will in turn result in a conformational change in gp16, which will produce the physical force. Site-directed mutagenesis results indicate that the conserved amino acids at the predicted Walker-A motif are important for the ATPase activity of gp16. The conserved amino acids G²⁴, G²⁹, K³⁰, and S³¹ may interact with β and γ phosphate through hydrogen bonding (HN··O=P).

Mutant gp16s with change(s) at the consensus amino acids of the Walker-A motif were inactive in phi29 DNA packaging but were still able to bind ATP (Table 2 and Fig. 5). This is not surprising since binding to ATP requires multiple residues to form an ATP-binding pocket, and the other unchanged, conserved amino acids could still hold the ATP. ATPase assays revealed that these mutants actually lost ATPase activity, suggesting that the amino acids in the Walker-A motif are involved not only in the binding for ATP, but also in ATP hydrolysis. Recently it was predicted that the Walker-B motif of gp16 is the sequence T⁷⁷I⁷⁸V⁷⁹F⁸⁰D⁸¹ (Mitchell et al., 2002). The validity of this prediction remains to be confirmed by the change of the acidic D⁸¹/E⁸² to other basic amino acids, following the approach used in this studies for Walker-A motif.

Materials and methods

ATP-binding assay using ATP-agarose affinity column

The gene for gp16 was fused with a 109 amino acid thioredoxin, a six-His-tag, and a 15 amino acid S-tag (Huang and Guo, 2003). Both His-tag and S-tag were placed between thioredoxin and gp16. The S-tag served as a marker for gp16 identification. The size of the resulted His-tagged gp16 is 54 kDa. It was purified by one-step Ni-agarose column to homogeneity (Huang and Guo, 2003).

A 0.8-cm-diameter column was packed with 1 ml ATP-affinity agarose resin (Sigma) (5 mM ATP immobilized through the C-8 position of the base to cyanogen bromide activated agarose). The lyophilized resin was soaked in distilled water for more than 30 min before column packing. After washing with 12 ml of distilled water and then with 12

ml of binding buffer (100 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 5% glycerol, 20 mM Tris-HCl, pH 7.8), 100 μ l of His-tagged gp16 (30 μ g) in binding buffer was applied to the ATP affinity column. The column was then washed with 7 ml of binding buffer and eluted with 3–5 ml of binding buffer containing 10 mM ATP. Fractions (0.5–1 ml/fraction) were collected and subjected to S-Tag dot blot assay (see below), 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), silver stain detection, or activity assay for in vitro phi29 assembly. Similar approaches were applied to other resins.

Double-reciprocal plotting K_d determination

His-tagged gp16 was attached to the His-resin to form a gp16/His-resin complex. Thirty microliters of the complex was transferred into each Microcon YM-30 (Millipore), equilibrated with ATP-binding buffer, and then spun at 4°C to remove the liquid. Binding buffer (150 μ l) containing different concentrations of ATP, which were mixed with [α -³²P]ATP (Amersham), ranging from 0.8 μ M to 500 μ M, were added to the gp16-enriched His-resin in Microcons. After mixing and 5-min incubation, the mixture was spun for 3 s at 14,000 rpm in a microcentrifuge to separate the bound and unbound ATP. The pass-through liquid was collected. Twenty microliters of each aliquot was applied to a liquid scintillation counter. In the double-reciprocal plotting method, the equation ($r = K_d/[ATP] + 1$) was used. $1/r$ versus $1/[ATP]$ gives a straight line, and the slope of this line represents the K_d . r is defined as the amount of ATP bound to 1 mole of His-tagged gp16, and $r = [ATP]_{\text{bound}}/[gp16]_{\text{total}}$.

Construction of site-directed mutant gp16s

A site-directed mutagenesis kit (QuickChange, Stratagene) was used to make point mutations. Both primers containing the desired mutation were annealed to the methylated plasmid PART7ED that contained the gp16 gene and polymerized by the high-fidelity DNA polymerase Pfu-Turbo. *DpnI* digestion was performed to remove the methylated, nonmutated parental DNA template. The resultant circular, nicked dsDNA plasmids were transformed into *E. coli* XL1-Blue supercompetent cells. Mutations were confirmed by DNA sequencing. These mutants were then subcloned into the pET-32 vector to produce His-tag fusion gp16. Mutant His-tagged gp16s were then purified using Ni-agarose column chromatography as described above.

Detection of His-tagged gp16 by S-tag Western blot

A dot-blotting apparatus (Millipore) was used to transfer protein samples to an Immobilon-P transfer membrane. The membranes were pretreated with blocking buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, and 1% gelatin) for 15 min. The membranes were then incubated for

another 15 min in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing S-protein alkaline phosphatase conjugate. After washing four times with TBST buffer, the blots were incubated for 15–30 min in the alkaline phosphatase buffer containing the substrates NBT/BCIP for color development.

ATPase assay by thin-layer chromatography (TLC)

His-tagged gp16 or mutants (G29S&K30I, R17E, G24D, and G27D) (0.2 mg/ml), DNA-gp3 (0.1 μ g), procapsid (10 μ g), and RNA (200 ng) were assayed individually and in combination for ATPase activity. When one or more constituents were omitted, they were replaced with the appropriate volume(s) of TMS buffer (100 mM NaCl, 10 mM MgCl₂, 50 mM Tris–HCl, pH 7.8). The reaction mixture contained 0.1 mM of unlabeled ATP and 30 μ Ci/ml of [γ -³²P]ATP (Guo et al., 1987b). After 30 min of incubation at room temperature, 2 μ l of the reaction mixture was spotted onto a PEI-cellulose plate (J.T. Chemical Co.) and air-dried. The plates were then soaked in methanol for 5 min, air-dried, and run in 1 M formic acid and 0.5 M lithium chloride.

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