The ATPase of the phi29 DNA packaging motor is a member of the hexameric AAA+ superfamily

Chad Schwartz¹, Gian Marco De Donatis¹, Huaming Fang¹, Peixuan Guo*  

Nanobiotechnology Center, College of Pharmacy and Markey Cancer Center, University of Kentucky, Lexington, KY, USA

ARTICLE INFO

Keywords: Revolution Motor Hexameric ATPase Nanobiotechnology Nanomotor Push through a one-way valve

ABSTRACT

The AAA+ superfamily of proteins is a class of motor ATPases performing a wide range of functions that typically exist as hexamers. The ATPase of phi29 DNA packaging motor has long been a subject of debate in terms of stoichiometry and mechanism of action. Here, we confirmed the stoichiometry of phi29 motor ATPase to be a hexamer and provide data suggesting that the phi29 motor ATPase is a member of the classical hexameric AAA+ superfamily. Native PAGE, EMSA, capillary electrophoresis, ATP titration, and binomial distribution assay show that the ATPase is a hexamer. Mutations in the known Walker motifs of the ATPase validated our previous assumptions that the protein exists as another member of this AAA+ superfamily. Our data also supports the finding that the phi29 DNA packaging motor uses a revolution mechanism without rotation or coiling (Schwartz et al., this issue).

Introduction

The superfamily of AAA+ motors (ATPases associated with diverse cellular activities) plays a key role in several assorted functions, and many members of this clade of ATPases often fold into hexameric structures (Mueller-Cajar et al., 2011; Wang et al., 2011). Despite their diversity, the common characteristic of this family is their ability to convert chemical energy from the hydrolysis of the γ-phosphate bond of ATP into a protein conformational change. This change of conformation generates a loss of affinity for its substrate and exerts a mechanical movement, which in turn is used to either make or break contacts between macromolecules, resulting in local or global protein unfolding, assembly or disassembly of complexes, or transport of macromolecules relative to each other. These activities underlie processes critical to DNA repair, replication, recombination, chromosome segregation, dsDNA transportation, membrane sorting, cellular reorganization, and many other functions (Martin et al., 2005; Ammelburg et al., 2006).

DsDNA viruses package their DNA genome into a preformed protein shell called a procapsid, with the aid of a nanomotor (Feiss and Rao, 2012; Guo and Lee, 2007; Fang et al., 2012; Zhang et al., 2012). Since 1978, it has been popularly believed that viral DNA packaging motors run through a five-fold/six-fold mismatch rotation mechanism (Hendrix, 1978). An RNA component (pRNA) was discovered on the phi29 DNA packaging motor (Guo et al., 1987a), and subsequently, pRNA was determined to exist as a hexameric ring (Guo et al., 1998; Zhang et al., 1998). Based on this structure, it was proposed that the mechanism of the phi29 viral DNA packaging motor is similar to that used by other hexameric DNA tracking motors of the AAA+ family of proteins (Guo et al., 1998). A debate subsequently developed concerning whether the RNA and ATPase of the motor exist as hexamers (Zhang et al., submitted for publication, 2012, 1998; Guo et al., 1998; Shu et al., 2007; Xiao et al., 2008; Ibarra et al., 2000) or as pentamers (Moffitt et al., 2009; Yu et al., 2010; Chistol et al., 2012). The differing viewpoints have not yet been fully reconciled, but we have recently shown using X-ray diffraction, AFM imaging, and single molecule studies that the motor consists of three coaxial rings geared by hexameric pRNA (Zhang et al., submitted for publication) (Fig. 1). The force generation mechanism of the phi29 DNA packaging motor is still under debate (Moffitt et al., 2009; Aathavan et al., 2009; Jing et al., 2010; Zhang et al., 2012; Geng et al., 2011; Fang et al., 2012; Schwartz et al., 2012).

The phi29 DNA packaging motor reconstituted in the de

* Correspondence to: William Farish Endowed Chair in Nanobiotechnology, School of Pharmacy, University of Kentucky, 565 Biopharmaceutical Complex, 789 S. Limestone Street, Lexington, KY 40536, USA.  
E-mail address: peixuan.guo@uky.edu (P. Guo).  
1 These authors contributed equally.

Published by Elsevier Inc.

Please cite this article as: Schwartz, C., et al., The ATPase of the phi29 DNA packaging motor is a member of the hexameric AAA+ superfamily. Virology (2013), http://dx.doi.org/10.1016/j.virol.2013.04.004

http://dx.doi.org/10.1016/j.virol.2013.04.004
The phi29 DNA packaging motor contains three coaxial rings

The phi29 DNA packaging motor consists of three major structural components: the connector, pRNA, and ATPase gp16 (Fig. 1). Extensive studies (Guo, 2002; Green et al., 2010; Ibarra et al., 2000; Xiao et al., 2008; Zhang et al., 1998; Shu et al., 2007; Trottier and Guo, 1997; Chen et al., 1997) of the pRNA and a recent crystal structure (Zhang et al., 2013) has revealed that pRNA exists as a hexamer, as also confirmed by AFM (Shu et al., in press). These data show that the three coaxial rings are connected to each other with fixed stoichiometry.

Native PAGE, EMSA, and CE reveal hexameric ATPase

Fusion of eGFP to the N-terminus of gp16 results in fluorescent gp16 (eGFP-gp16) that shows similar biological activity as native gp16 (Lee et al., 2009). eGFP-gp16 yields six distinct fluorescent
bands on a native PAGE gel which separates solely on the basis of mass (see Materials and methods), indicative of six monomers oligomerizing to form a hexamer (Fig. 2A). The monomer and all even numbered oligomer bands have a higher intensity than the trimer and pentamer, suggesting that the assembly sequence is monomer to dimer, to tetramer and finally to hexamer, such that the final gp16 oligomeric state is likely a trimer of dimers, as in other ATPases (Sim et al., 2008; Skordalakes and Berger, 2006; Ziegelin et al., 2003; Sim et al., 2008). In addition, as the concentration of gp16 is increased, the intensity of the hexamer band increased significantly, while the intensity of smaller oligomers remains fairly constant, further suggesting that a hexamer is the final oligomeric state. Finally, the presence of eGFP-gp16 hexamer was further confirmed by stoichiometric ratio assays as discussed in the following sections.

Electrophoretic mobility shift assays (EMSA) were employed with the fluorescent eGFP-gp16 and with a short 40 bp dsDNA fragment conjugated to a Cy3 fluorophore. The two components were mixed together with ATP and a non-hydrolyzable ATP analog (γ-S-ATP) (Fig. 2B). The ATPase bound more tightly to the dsDNA upon addition of γ-S-ATP (Fig. 2B, lane 6) as observed previously (Schwartz et al., 2012). Furthermore, after addition of ATP to the gp16:DNA complex, two distinct ATPase bands were present (Fig. 2B, lanes 7,8), perhaps representative of two different conformational states of gp16.

We repeated the EMSA with increasing amounts of ATPase and a fixed amount of dsDNA to determine the stoichiometry of the ATPase bound to dsDNA. As the molar concentration ratio of gp16:dsDNA reaches 6:1, free dsDNA (bottom band, Fig. 3A Cy3 channel) shifted nearly entirely to the bound state (top yellow band, lane 6). Capillary electrophoresis was used to validate the qualitative EMSA data. In this case, the amount of gp16 was held at 3 μM, mainly due to the stickiness of the protein in the small capillary, and the [dsDNA] was varied in the reaction mixture. The fluorescent peak corresponding to the DNA:protein complex was quantified over a range of dsDNA concentrations. A plateau was achieved at 0.5 μM DNA bound, representing a ratio of DNA:protein of 1:6, further arguing that the gp16 ATPase is a hexamer (Fig. 3B).

Mutations of known motifs suggest that phi29 gp16 is a member of the AAA+ superfamily of ATPases

Gp16 contains well-conserved motifs responsible for ATP binding (Walker A and Arginine finger) and ATP hydrolysis (Walker B), typical of all AAA+ proteins. The Walker A motif was previously identified, but the Walker B motif had not been determined (Guo et al., 1987c). Sequence alignment with AAA+ proteins revealed the Walker B motif (hhhhDE) at residues 114–119 (TIVFDE), where ‘h’ represents hydrophobic residues.

To confirm the results of sequence alignment, relevant amino acids of both Walker A and Walker B motifs were mutated. For the Walker A motif, the previous mutant G27D was cloned. In the Walker B motif, two mutants were generated: E119A and D118E/S.

Fig. 2. (A) 6% Native PAGE using a non-denaturing detergent which fractionates by size reveals distinct bands characteristic of six oligomeric states; the top, hexameric band increased as the concentration of protein is increased (15 μM, 17.5 μM, 20 μM). Oligomeric states were assigned based on the mobility of marker proteins in the Native PAGE Mark kit. (B) EMSA of native eGFP-gp16 (3 μM) with short, 40 bp Cy3-dsDNA (300 nM) and ATP (30 nM) or γ-S-ATP (1.25 mM). The GFP channel (left) shows migration of the fluorescent eGFP-gp16 and with a short 40 bp dsDNA fluorophore. The two components were mixed together with ATP and a non-hydrolyzable ATP analog (γ-S-ATP) (Fig. 2B). The ATPase bound more tightly to the dsDNA upon addition of γ-S-ATP (Fig. 2B, lane 6) as observed previously (Schwartz et al., 2012). Furthermore, after addition of ATP to the gp16:DNA complex, two distinct ATPase bands were present (Fig. 2B, lanes 7,8), perhaps representative of two different conformational states of gp16.

Please cite this article as: Schwartz, C., et al., The ATPase of the phi29 DNA packaging motor is a member of the hexameric AAA+ superfamily, Virology (2013), http://dx.doi.org/10.1016/j.virol.2013.04.004
E119D double mutant. The most important residues in Walker B are the aspartate (D) for its role in magnesium ion binding, and glutamate (E) responsible for the activation of a water molecule to perform a nucleophilic attack on the gamma phosphate of ATP. Both mutants were tested for their ability to hydrolyze ATP and to bind DNA.

Both mutants were subjected to the ATP hydrolysis assay (Lee et al., 2008). Only the wildtype ATPase hydrolyzed ATP (Fig. 4A); the Walker A G27D mutant is incapable of binding ATP while the Walker B mutant can bind, but cannot hydrolyze. We expanded our testing of the mutants in terms of DNA binding. Using the same capillary electrophoresis assay used for wildtype ATPase, we quantified the DNA bound peak of both mutants. In the presence of γ-S-ATP, the wildtype and Walker B mutant displayed similar DNA binding affinities. However, upon addition of ATP, the wildtype no longer remains bound to DNA as previously shown (Schwartz et al., 2012), but the Walker B D118E/E119D mutant retains its DNA binding capability, suggesting that this identified motif is in fact responsible for the catalytic step which pushes dsDNA away from gp16 upon hydrolysis.

Lastly, we attempted to validate our findings using EMSA (Fig. 4C). Gp16 ATPase and fluorescent DNA were mixed together and incubated at room temperature for 20 min. The samples were then loaded into an agarose gel. The top, green gel represents the fluorescent signal of the eGFP-conjugated ATPase; however, the bottom, yellow gel shows the migration of the cy3-fluorescent dsDNA. In the cy3 gel, the upper bands are representative of DNA bound to gp16 ATPase as the protein retards the migration of the short DNA. However, the bottom bands are free DNA as the negatively-charged strand of nucleotides quickly migrates to the positive electrode. Again, the wildtype gp16 ATPase exhibits high affinity to dsDNA with addition of γ-S-ATP (lane 3), but diminished affinity with ATP or no phosphate analog (lanes 2,4). The Walker A G27D mutant has diminished binding affinity in all cases (lanes 5–7), albeit higher affinity with addition of γ-S-ATP, as this mutant is incapable of binding ATP which stabilizes the interaction between gp16 and dsDNA. Finally, the Walker B D118E/E119D mutant which previously has been shown to be incapable of hydrolyzing ATP, was incapable of binding without ATP (lane 8), but exhibited high affinity with both ATP and γ-S-ATP (lanes 9,10).
Both the capillary electrophoresis quantification and the EMSA confirmed our hypothesis that the recently discovered Walker B motif of phi29 ATPase is responsible for ATP hydrolysis.

**Discussion**

For many years, there has been a debate as to the stoichiometry of phi29 motor components. Many data has been shown by both camps as to the five-fold and six-fold nature of the gp16 ATPase and packaging RNA. However, both sides are in agreement that the stoichiometries of these two components exist in a 1:1 ratio. It has previously been shown that the symmetry remains uniform between the ATPase and pRNA whether it exists as a pentameric pRNA and pentameric ATPase or hexameric pRNA and hexameric ATPase, suggesting that the two work in unison, independent of the stoichiometry.

The data shown here indicate that gp16 ATPase is a member of the AAA+ superfamily of proteins, and similar to this family, the phi29 motor ATPase also exists in either a high or low affinity state for DNA substrate. Recently, it has been qualitatively demonstrated via EMSA (Schwartz et al., 2012) that the ATPase gp16 is capable of binding to dsDNA in the presence of γ-S-ATP. Fusion of a fluorescent tag on the ATPase did not affect its function or activity (Lee et al., 2009), but provided a marker for binding assays. In the previous reports, a small amount of Cy3-dsDNA was bound by eGFP-gp16 using the EMSA. However, stronger binding of gp16 to dsDNA was observed when gp16 was incubated with γ-S-ATP and dsDNA (Schwartz et al., 2012). Also in the previous reports, Förster Resonance Energy Transfer (FRET) analysis and sucrose sedimentation studies further validated our finding that the gp16/dsDNA complex is stabilized by addition of γ-S-ATP (Schwartz et al., 2012). Furthermore, the data confirmed that gp16 possesses both a DNA binding domain and a Walker A motif with which to bind ATP (Schwartz et al., 2012).

By sequence homology and point mutation analysis, both the Walker A and Walker B motifs have been shown to be involved in ATP hydrolysis in the ATPase of phi29 (Guo et al., 1987a; Huang and Guo, 2003a). As expected, all the mutants were severely impaired in ATP hydrolysis activity and were similar to the Walker A mutant G27D, proving that the Walker A motif is responsible for binding of ATP. Regarding the ability to bind to DNA in the presence of γ-S-ATP, mutations in the walker A motif displayed a limited ability to bind DNA compared with the wild-type (Fig. 4B, C), most likely due to their impaired affinity for γ-S-ATP. On the contrary, the walker B mutants retained their binding affinity for DNA in the presence of γ-S-ATP and were also sufficient to bind DNA in the presence of ATP, confirming that the Walker B mutation only affects the ability to hydrolyze ATP but not the binding to the nucleotide.

Our data shows that in the absence of ATP, or its derivative γ-S-ATP, the binding of gp16 to DNA is reduced. However, after the addition of γ-S-ATP the binding efficiency of gp16 to DNA increased significantly (Fig. 4B, C). This suggests that ATP induces a change in gp16 that causes it to assume a high affinity conformation for dsDNA binding, a conclusion strengthened by the inability of the Walker A mutant protein, which cannot bind ATP, to elicit a conformational change. In the previous report, when ATP was added to the gp16-γ-S-ATP-dsDNA complex, rapid ATP hydrolysis was observed (Schwartz et al., 2012) and gp16 dissociated from the dsDNA. This indicates that after hydrolysis, gp16 undergoes a further conformational change that produces an external force against the dsDNA that pushes the substrate away from the motor complex by a power stroke. This phenomenon can be seen in Fig. 2B in which the ATPase exists as two states after addition of ATP: DNA bound or expelled. However, introducing a mutation to the Walker B motif eliminated the catalytic force step. The data correlates nicely with other reports that Walker B mutants do not hydrolyze ATP, but bind strongly to DNA.

Gp16 is a DNA-dependent ATPase of the phi29 DNA packaging motor (Guo et al., 1987c; Huang and Guo, 2003a,b; Ibarra et al.,...
Energy is provided to the motor through ATP. As aforementioned, non-hydrolyzable γ-S-ATP stalled and fastened the gp16/dsDNA complex. It has been found that the hydrolysis of ATP leads to the release of dsDNA from gp16. After ATP was added to the gp16/dsDNA/γ-S-ATP complex, the band representing the gp16/dsDNA complex disappeared (Schwartz et al., 2012). The release of dsDNA from the gp16/dsDNA/γ-S-ATP complex by ATP was also demonstrated by sucrose gradient sedimentation (Schwartz et al., 2012). Hydrolysis of ATP was confirmed when the purified gp16/dsDNA/γ-S-ATP hydrolyzed ATP after the addition of ATP to the purified complex (Schwartz et al., 2012). These results suggested that hydrolysis of ATP leads to the release of dsDNA from the gp16, forcing the DNA substrate away from the interior pocket of the ATPase, and lending to physical motion of genomic DNA towards the capsid.

Our data combining the stoichiometry of the ATPase and the sequential action previously elucidated (Schwartz et al., 2012), allows us to build upon our previous “push through a one-way valve” DNA packaging model. After binding to ATP, the ATPase undergoes a conformational change which significantly increases its affinity to dsDNA. An additional conformational change of the ATPase after release of inorganic phosphate causes gp16 to perform a power stroke to push dsDNA into the portal protein (Fig. 6).

The stoichiometry of the phi29 DNA packaging motor has long been a contentious subject. Here we have provided additional biochemical data showing that the ATPase gp16 consists of six subunits (Fig. 2A), upon binding to dsDNA (Fig. 3), and also in the active phi29 motor (Fig. 5). Furthermore, we have identified the classical Walker motifs typical of the hexameric AAA+ superfamily, and found that phi29 DNA packaging motor uses a revolution without rotation and coiling or generation of torque (Schwartz et al., this issue). In our accompanying paper in this issue, we show that the ATPase “hands off” the substrate dsDNA in a sequential action manner lending to revolution around the ATPase and connector protein. Our data leads to the conclusion that the hexameric stoichiometry and the mechanism of revolution for phi29 DNA packaging motor are in accordance with FtsK of the hexameric AAA+ superfamily, and we expect that most phages follow this “push through a one-way valve” via revolution mechanism (Zhao et al., in press; Schwartz et al., this issue).

Materials and methods

Cloning, mutagenesis and protein purification

The engineering of eGFP-gp16 and the purification of the gp16 fusion protein have been reported previously (Lee et al., 2009). The eGFP-gp16 mutants G27D, E119A, and D118E E119D were constructed by introducing mutations in the gp16 gene (Keyclon Technologies).

Measurement of gp16 ATPase activity

Enzymatic activity via fluorescent labeling was described previously (Lee et al., 2008). Briefly, a phosphate binding protein conjugated to a fluorescent probe that senses the binding of phosphate was used to assay ATP hydrolysis.

In vitro virion assembly assay

Purified in vitro components were mixed and were subjected to the virion assembly assay as previously described (Lee and Guo, 1994). Briefly, newly assembled infectious virions were inoculated with Bacillus bacteria and plated. Activity was expressed as the number of plaques formed per volume of sample (pfu/mL).

Statistical analysis and data plotting

Most statistical analysis was performed using Sigmaplot 11. The Hill coefficient was determined by nonlinear regression fitting of the experimental data to the following equation: 

\[ E = E_{\text{max}} \times (x^n) / (K_{\text{app}}+ (x^n)) \]

where E and E_{\text{max}} refer to the concentration of the gp16/dsDNA complex, X is the concentration of ATP or ADP, K_{\text{app}} is the apparent binding constant, and n is the Hill coefficient.

CE experiments to determine ratio of gp16 to bound dsDNA

CE (capillary electrophoresis) experiments were performed on a Beckman MDQ system equipped with double fluorescence detectors (at 488 nm and 635 nm excitation wavelength). A bare borosilicate capillary with a total length of 60 cm and a 50 μm inner diameter was used. Assay conditions contained separation
buffer of 50 mM Tris–HCl, 100 mM sodium borate at pH 8.00, 5 mM MgCl₂, 10% PEG 8000 (w/v), 0.5% acetone (v/v), 3 μM eGFP-gp16 monomer, and variable amounts of ATP/ADP and DNA.

Native PAGE of eGFP-gp16

Increasing amounts of eGFP-gp16 were loaded onto a 6% tris–glycine polyacrylamide gel in conjunction with the Native PAGE Mark kit (Invitrogen). This commercially available Native PAGE Mark kit uses a non-denaturing detergent to mildly solubilize and coats the protein with a negative charge. Thus, gel electrophoresis separates solely on the basis of mass. The gel was imaged using a Typhoon gel image scanner at an excitation wavelength of 488 nm.

Atomic force microscopy (AFM) imaging

APS mica was obtained by incubation of freshly cleaved mica in 167 mM 1-(3-aminopropyl) silatrane as described (Shlyakhtenkov et al., 2003; Lyubchenko and Shlyakhtenkov, 2009). Native PAGE purified RNA samples were diluted with 1X TBS buffer to a final concentration of 3–5 nM. Then, 5–10 μL of RNA was immediately deposited on the APS mica surface. After 2 min incubation, excess samples were washed with DEPC treated water and dried under a flow of Argon gas. AFM images in air were acquired using MultiMode AFM NanoScope IV system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode. Two types of AFM probes were used for tapping mode imaging in air: (1) regular tapping Mode Silicon Probes (Olympus from Asylum Research, Santa_Bara, CA) with a spring constant of ~42 N/m and a resonant frequency between 300 kHz and 320 kHz. (2) non-contact NSG01_DLC probes (K-Tek Nanotechnology, Wilsonville, OR) with a spring constant of about 5.5 N/m and a resonant frequency between 120 kHz and 150 kHz.

Electrophoretic mobility shift assay (EMSA)

The fluorescently tagged protein that facilitates detection and purification was shown to possess similar assembly and packaging activity as compared to wildtype (Lee et al., 2009; Schwartz et al., 2012). Cy3-dsDNA (40 bp) was prepared by annealing two complementary DNA oligos containing a Cy3 label (IDT) at its 5′ end and purified by a 10% polyacrylamide gel. Samples were prepared in 20 μL buffer A (20 mM Tris–HCl, 50 mM NaCl, 15% glycerol, 0.1 mM MgCl₂). Specifically, 1.78 μM eGFP-gp16 was mixed with 7.5 ng/μL of 40 bp Cy3-DNA in the presence or absence of ATP and γ-S-ATP. Samples were incubated at ambient temperature for 20 min and then loaded onto a 1% agarose gel (44.5 μM Tris, 44.5 mM boric acid) and electrophoresed at 4 C for 1 h at 8 V/cm. The eGFP-gp16 and Cy3-DNA samples were analyzed by a fluorescent LightTools Whole Body Imager using 488 nm and 540 nm excitation wavelengths for GFP and Cy3, respectively.

Acknowledgments

We would like to thank Dr. Guo-Min Li for his valuable comments; Yi Shu, Luda Shlyakhtenkov, and Yuri Lyubchenko for the AFM images of RNA polymerase; Zhengyi Zhao, Emil Khisamutdinov, and Hui Li for their diligent work on the animation figures; and Jeanne Haak for editing this manuscript. The work was supported by NIH Grants R01 EB012135, and U01 CA151648 to PG, who is a co-founder of Kylin Therapeutics, Inc., and Biomotor and Nucleic Acids Nanotech Development, Ltd.

Appendix A. Supporting information

Supplementary materials associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.04.004.

References


