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Interaction of gp16 with pRNA and DNA for Genome Packaging by the Motor of Bacterial Virus phi29

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One striking feature in the assembly of linear double-stranded (ds) DNA viruses is that their genome is translocated into a preformed protein coat *via* a motor involving two non-structural components with certain characteristics of ATPase. In bacterial virus phi29, these two components include the protein gp16 and a packaging RNA (pRNA). The structure and function of other phi29 motor components have been well elucidated; however, studies on the role of gp16 have been seriously hampered by its hydrophobicity and self-aggregation. Such problems caused by insolubility also occur in the study of other viral DNA-packaging motors. Contradictory data have been published regarding the role and stoichiometry of gp16, which has been reported to bind every motor component, including pRNA, DNA, gp3, DNA-gp3, connector, pRNA-free procapsid, and procapsid/pRNA complex. Such conflicting data from a binding assay could be due to the self-aggregation of gp16. Our recent advance to produce soluble and highly active gp16 has enabled further studies on gp16. It was demonstrated in this report that gp16 bound to DNA non-specifically. gp16 bound to the pRNA-containing procapsid much more strongly than to the pRNA-free procapsid. The domain of pRNA for gp16 interaction was the 5′/3′ paired helical region. The $C_{18}C_{19}A_{20}$ bulge that is essential for DNA packaging was found to be dispensable for gp16 binding. This result confirms the published model that pRNA binds to the procapsid with its central domain and extends its 5′/3′ DNA-packaging domain for gp16 binding. It suggests that gp16 serves as a linkage between pRNA and DNA, and as an essential DNA-contacting component during DNA translocation. The data also imply that, with the exception of the $C_{18}C_{19}A_{20}$ bulge, the main role of the 5′/3′ helical double-stranded region of pRNA is not for procapsid binding but for binding to gp16.

Keywords: bacteriophage phi29; DNA packaging; gp16; pRNA; procapsids; nanotechnology

Introduction

All linear double-stranded (ds) DNA viruses, including bacteriophages, adenoviruses, poxviruses and herpesviruses,[1–4] possess a common maturation feature in that their genome is translocated into a limited space within a preformed protein shell, known as a procapsid, to near-crystalline density.[3,5,6] This energetically unfavorable DNA motion process is accomplished by an ATP-driven motor.[2] Factors involved in the DNA-packaging motor and its components have the potential to be used as building blocks in nanotechnology.[7–9] This essential viral replication step has been investigated in the quest for model targets for the inhibition of viral replication[10,11] and for the development of drugs against cytomegalovirus (CMV).[12]

An examination of the well-studied dsDNA viruses reveals that the motor involves a dodecameric protein connector[13–16] and two non-structural components with certain characteristics typical of an ATPase.[17] The larger subunit of these two

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Abbreviations used: ds, double-stranded; pRNA, packaging RNA; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; BSA, bovine serum albumin.

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non-structural components is for procapsid binding and the smaller subunit is for DNA binding.[17] Procapsid-binding components include gpA of phage lambda,[18–20] gp12 of phi21,[21] gp17 of T4[22] and gp19 of T3/T7.[23,24] Components interacting with DNA include gpNu1 of lambda,[25,26] gp1 of phi21,[21] gp3 of phi29 and gp18 of T3/T7.

Emerging information reveals that the DNA-packaging mechanisms of CMV and adenovirus are very similar to those of phages. Herpes simplex virus contains a pair of DNA-packaging proteins, UL15 and UL28.[27] CMV also contains a pair of proteins, pUL56 and pUL89,[28] while the pair for adenovirus is the IVa2 and L1 52/55 kDa proteins.[29–33] The two non-structural motor components of phage lambda form a hetero-oligomer named terminase composed of gpNu1, which binds cooperatively to three DNA recognition sites within *cosB*, and gpA, which, in addition to its capability for connector binding, binds to *cosN*, at which site the duplex is nicked and separated.[18,34] Genome packaging begins with terminase assembly at *cos*, which is the packaging initiation site in the DNA concatemer, then it recruits an empty procapsid, which initiates ATP-powered translocation of the packaging machinery along the duplex, thus packaging DNA.[18,34] It has been reported that the recombinant lambda terminase forms a hetero-trimeric structure consisting of one gpA and two gpNu1 proteins; this has been confirmed by studies of sedimentation velocity, sedimentation equilibrium and gel filtration chromatography.[35] The stoichiometry of the interaction between g1p and g2p in SPP1 has been indirectly investigated by monitoring the stimulation of the ATPase activity of g2p upon addition of g1p. It has been proposed that the terminase complex consists of two or three ring-shaped decamers of g1p associated with a monomer of g2p, and the portal complex, a dodecamer of the g6p protein.[36] In the T4 system, it has been reported that the packaging protein, gp16, preferentially binds dsDNA containing a gene *16* coding region,[37] and stimulates the formation of higher-order oligomers of the large terminase subunit (gp17).[38] These oligomers have stimulated ATPase activity. In bacteriophage T3, the interaction of the larger DNA-packaging component gp19 with the connector has been demonstrated. Six molecules of gp19 bound to the procapsid with a saturating amount of gp19, but the gp19 did not bind to the procapsid lacking the connector (the gp8-deficient procapsid).[24] For lambda, it has been found that the target component of gpA binding is the connector protein, since studies with the gpA and connector double mutant revealed that a connector mutation can suppress a gpA mutation that hinders gpA binding to procapsid.[19,20]

The phi29 DNA-packaging motor also involves nonstructural proteins. Terminal protein gp3, which covalently links to each 5′ end of the genomic DNA, is indispensable for DNA packaging.[39,40] The other non-structural protein, gp16, has been reported to be the critical component in DNA packaging and is a DNA and procapsid-dependent ATPase.[17,41] A significant novelty of the phi29 DNA-packaging motor is that the motor involves an RNA molecule,[42] named pRNA, that forms a hexameric ring[43–46] and is attached to the N terminus of the connector protein gp10.[47] Originally, it was proposed that gp3 and gp16 were non-structural DNA-packaging proteins. gp3, with its smaller molecular mass, was believed to be responsible for DNA binding, and the larger subunit, gp16, which contained the A-type and B-type sequences for ATP-binding, was believed to be responsible for procapsid binding.[17] However, our recent finding that pRNA binds ATP,[48] interacts with the procapsid, and connects to the N terminus of connector protein gp10 has led to the re-categorization of pRNA and gp16 with regard to the pair of non-structural DNA-packaging proteins.[45] Accumulated data have conclusively confirmed that pRNA is the procapsid-binding component.[42,49–51] This has led to the speculation that pRNA is the counterpart of gpA of lambda and gp19 of T3,[47] since pRNA, gpA and gp19 all bind to the connector and contain the ATP-binding motif.[48,52–55] The re-categorization of pRNA as the larger subunit of the pair of the non-structural proteins has raised the question of whether the main role of gp16 is DNA binding.

As noted earlier, the phi29 motor involves three essential components: the 12 subunit connector,[13–16] the six subunit pRNA hexamer[43–46], and a protein, gp16. The crystal structure of the connector has been solved,[13,16] and three-dimensional models of pRNA monomer, dimer, hexamer and the connector/pRNA complex have been reported.[56] It has been found that pRNA contains two functional domains. The procapsid-binding domain binds to the N terminus of connector protein gp10.[47] However, the role of the DNA-packaging domain located at the 5′/3′ paired helical region of pRNA remains unknown. In addition, studies on the role of gp16 in the motor have been significantly hampered by both the insolubility of DNA-packaging protein gp16 after expression and by self-aggregation after purification. Little progress has been made since the finding of ATPase activity and the overproduction and purification of the gene product of gp16.[41] In that traditional method, gp16 was purified in a denatured condition, and active gp16 was obtained by dialysis against 4 mM KCl buffer for 40 min for renaturation. The re-natured gp16 aggregated again within 15 min of re-naturation. Contradictory data regarding ATPase activity, binding location, and the stoichiometry of gp16 have been published.[17,49,57,58] It was also reported that gp16 bound to procapsid before DNA packaging,[17] However, at that time, pRNA of the phi29 motor had not been discovered and there was no indication that pRNA was required for the binding of gp16 to the procapsid.[42] More recently, the interaction of gp16 with procapsid was investigated, and it was reported that gp16 bound to the procapsid, whether pRNA was

present or absent,[40,58] and competed with pRNA for procapsid binding.[58] However, within the same paper, it was also reported that gp16 bound to pRNA through a filter binding assay.[58] These discrepancies have arisen due to the lack of a method to make gp16 completely soluble. Due to aggregation, it is not surprising to find that gp16 binds to every motor component, including pRNA, DNA-gp3, pRNA-free procapsid and pRNA-containing procapsid.[40,57,58] It was reported that gp16 was made soluble in the cell by co-expression with chaperonin GroEL and its co-chaperonin GroES.[58] Indeed, active gp16 was purified using this overexpression system. Although co-expression with GroEL/ES solved the problem of aggregation in the cell, it could not solve the problem of self-aggregation after purification.

Recently, we have developed novel approaches to produce soluble and highly active recombinant gp16 by fusing gp16 with thioredoxin and attaching a His-tag to its N terminus and keeping gp16 in polyethylene glycol (PEG) and/or acetone.[59,60] This has resulted in a soluble and stable gp16 with an increase in activity of several thousand-fold.[59,60] This achievement has enabled the further refinement of the role of gp16 in the phi29 DNA-packaging motor. This paper focuses on the study of the interaction of gp16 with pRNA and DNA. It was found that pRNA was needed for the binding of gp16 to procapsid and that gp16 bound to the 5′/3′ paired DNA-packaging domain of pRNA. It was also found that gp16 binds to DNA non-specifically. These results suggest that gp16 functions as a linker between pRNA and DNA, and thereby serves as an essential DNA-contacting component during DNA translocation using the phi29 DNA-packaging motor.

Results

Studies on the interaction of gp16 with phi29 DNA-gp3

The interaction of gp16 and gp3 was investigated by flowing the [^{3}H]phi29 genomic DNA, with or without gp3, through an affinity column with gp16 immobilized on Ni^{2+}-NTA resin. After washing out of the unbound DNA–gp3, the bound DNA–gp3 was co-eluted with gp16 by the His-tag elution buffer, which removed gp16 from the column. No peak appeared under the same conditions when [^{3}H]phi29 DNA–gp3 was applied to a bare column without gp16, which indicated that gp16 binds phi29 DNA–gp3 (Figure 1). Furthermore, gp3-free phi29 DNA, pre-treated with proteinase K to remove gp3 before application into the gp16 affinity column, showed a binding pattern similar to phi29 DNA–gp3 (Figure 1), indicating that gp16 and DNA interaction was not affected by the presence of terminal protein gp3.

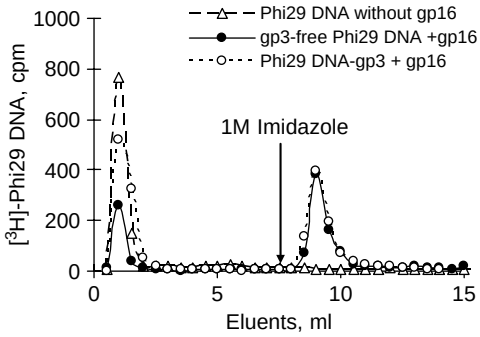

Figure 1. Binding of gp16 to phi29 DNA demonstrated with a gp16 affinity column. [^{3}H]phi29–gp3 DNA (filled circle) and gp3-free [^{3}H]phi29 DNA (open circle) were applied onto gp16 immobilized on Ni^{2+}-NTA resin. After washing, the bound DNA was co-eluted together with gp16 by 1 M imidazole in nucleic acid binding buffer.

Searching for the specific signal on phi29 DNA for interaction with gp16

gp3-free phi29 DNA and the phi29 DNA–gp3 complex were restricted by EcoRI, and 5 different-sized DNA fragments were produced. The EcoRI fragments A and C represented the left and right ends, respectively, of phi29 DNA that contained gp3 (Figure 2 (a) and (b)). EcoRI-digested fragments in the mixture were added to the gp16 in the affinity column. After elution by imidazole, all DNA fragments were located at similar fluent fractions, and no significant difference was observed between the gp3-containing fragments (A and C) and the gp3-free-fragments (B, D and E) on a gel (Figure 2 (c) and (d)), suggesting that gp16 can directly bind DNA in a non-sequence specific manner and is gp3-independent.

Competition binding assay to determine the specificity in gp16 and DNA interaction

A competition binding assay was performed to investigate the specificity of gp16 and DNA interaction. Four times the amount of unlabeled herring sperm DNA was added into [^{3}H]phi29 DNA–gp3 for competition binding to gp16 immobilized in affinity column. Binding efficiency was calculated by dividing the sum of cpm for eluted peak fractions by total cpm for every fraction. [^{3}H]phi29 DNA alone bound to gp16 with efficiency of 68.4% of total amount of tested DNA. However, [^{3}H]phi29 DNA mixed with unlabeled herring sperm DNA showed 18.5% binding efficiency, which was 3.7-fold lower efficiency compared to phi29 DNA alone (Figure 3). This means that unlabeled herring sperm DNA interferes the binding between phi29 DNA and gp16, implying that gp16–DNA interaction was non-specific.

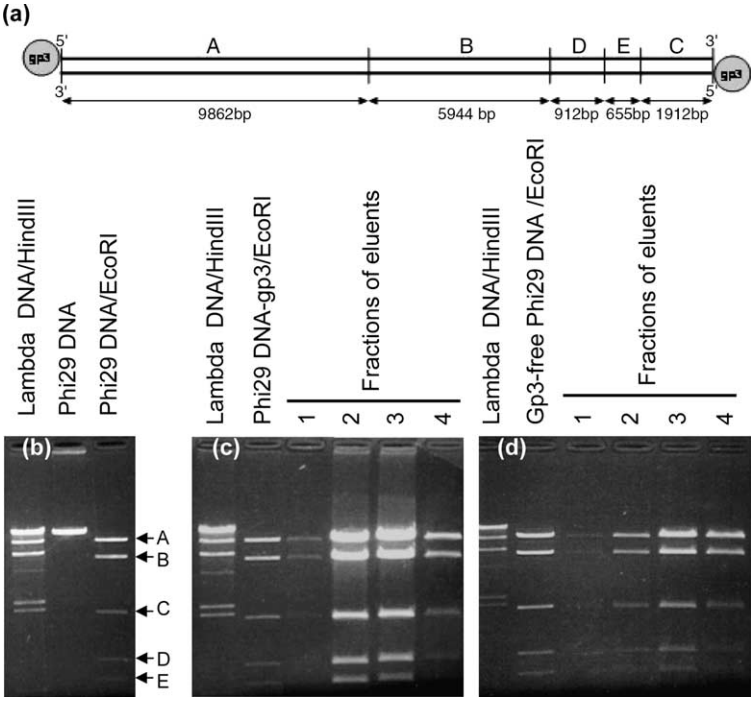

Figure 2. Binding of gp16 to phi29 DNA restriction fragments with or without gp3. (a) Schematic diagram of the EcoRI restriction map for phi29 DNA. The fragments A and C represented the left and right ends, respectively. (b) Unrestricted and restricted phi29 DNA EcoRI fragments analyzed on a 0.8% agarose gel. The restricted fragments of phi29 DNA–gp3 (c) and gp3-free DNA (d) were applied to a gp16 affinity column and, after washing, were eluted by 1 M imidazole in nucleic acid binding buffer. All DNA samples were treated with 1 mg/ml of proteinase K to remove gp3 before being analyzed on the 0.8% agarose gel.

Studies on the specificity of pRNA–gp16 interaction

A gp16 affinity column was used to investigate the specificity of gp16–pRNA interaction. [^{3}H]pRNA was applied to a gp16 affinity column. After washing the column with binding buffer to remove the unbound [^{3}H]pRNA, the column was eluted with 0.5 M NaCl in eluting buffer. We found that RNA can be eluted from the gp16 column at this salt concentration, while the gp16 immobilized to Ni^{2+}-NTA resin remained in the column for up to 2 M of NaCl. [^{3}H]pRNA showed strong binding to gp16, while the control experiment with [^{3}H]18 S RNA revealed that most 18 S RNA passed through the column before elution (Figure 4(a)), indicating

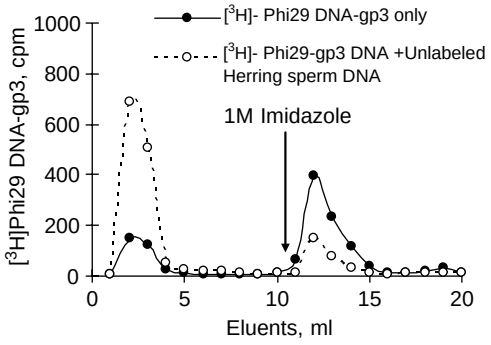

Figure 3. Competition assay for phi29 DNA binding to gp16. One μg of [^{3}H]phi29 DNA–gp3 with (filled circle) or without (open circle) 4 μg of unlabeled herring sperm DNA as a competitor, was applied onto a gp16 affinity column and co-eluted together with gp16 by 1 M imidazole.

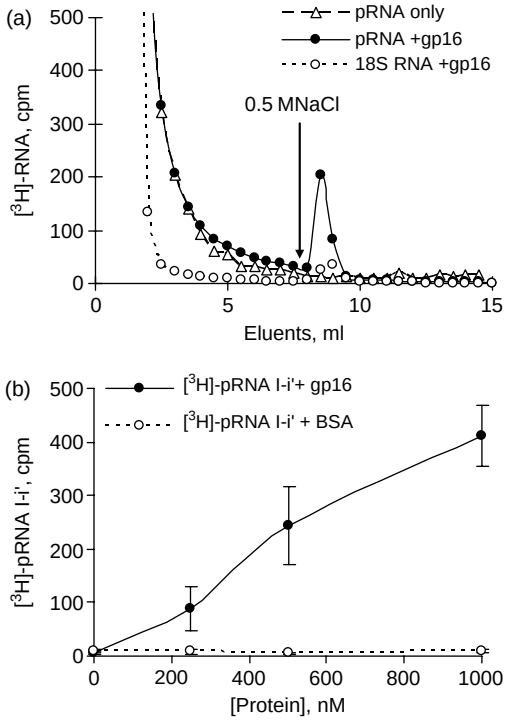

Figure 4. Binding specificity of gp16 to pRNA. (a) A total of 50 pmol each of [^{3}H]pRNA (filled circle) and [^{3}H]18 S RNA (open circle) were flowed through the gp16 affinity column and eluted by 0.5 M NaCl in nucleic acid binding buffer. To determine the background level of interaction between pRNA and the resin matrix, [^{3}H]pRNA was applied to a bare affinity column without gp16 (open triangle). (b) Filter binding assay for pRNA binding to gp16. [^{3}H]pRNA I-i′ was mixed with gp16 (filled circle) in nucleic acid binding buffer and applied to a glass fiber filter. After washing, the dried glass filter was subjected to liquid scintillation counting. Bovine serum albumin was used as a control (open circle).

that gp16 bound pRNA with higher specificity than other RNA.

In order to confirm that gp16 binds pRNA in a specific manner, [^{3}H]pRNA was mixed with gp16, and then the mixture was passed through a glass fiber filter. If radioactively-labeled pRNA binds to protein, pRNA attaches to the glass fiber filter due to the retention of the protein on the filter. [^{3}H]pRNA was attached to the glass fiber filter in proportion to the concentration of gp16, while [^{3}H]pRNA was not retained when added to bovine serum albumin (BSA) in the control experiment (Figure 4(b)). The results indicated that gp16 can bind specifically to pRNA.

Determination of whether pRNA is needed for the interaction of gp16 with procapsid

RNA-free procapsids were prepared from *Escherichia coli* cells that over-expressed the procapsid components gp7, gp8 and gp10.[61,62] In order to exclude the possibility of RNA contamination during procapsid preparation, procapsids were further purified by treatment with EDTA, which chelated Mg^{2+}, an ion essential for RNA binding to procapsids. After centrifugation to remove the remaining EDTA, no RNA contamination was observed, as demonstrated by gel electrophoresis (data not shown). Procapsids were applied to the gp16 affinity column, and the presence of procapsid in the eluents was detected by enzyme-linked immunosorbent assay (ELISA) using purified rabbit IgG against phi29 procapsid. It was shown that most pRNA-free procapsids passed through the column without significant binding to the gp16 affinity column. When procapsids were pre-incubated with pRNA before loading onto the column, the pRNA–procapsid complex was clearly detected in the fractions after elution by salt (0.5 M NaCl), indicating that gp16 bound to procapsids in the presence of pRNA (Figure 5). In contrast, the effect of pRNA on gp16 binding to procapsids was not duplicated with 18 S RNA (Figure 5), which suggested that gp16 bound to procapsids through specific intermediation with pRNA.

Investigation of the role of the DNA-packaging domain of pRNA for gp16 interaction

As noted above, pRNA contains two functional domains. The procapsid binding domain is located between nucleotides 23–97. The domain at the 5′/3′ paired ends is essential for DNA packaging;[63] however, the exact role of this domain is unknown. In order to examine the role of this domain, mutant pRNA I-i′ 23–97,[64] which is a deletion mutant with the 5′/3′ paired end (nt 1–22 and 98–120) removed while procapsid binding activity remained unchanged, was investigated on a gp16 affinity column. pRNA I-i′ 23–97 was added to procapsids to be a complex before loading to the gp16 affinity column, and was eluted by 0.5 M NaCl. The co-eluted procapsids were detected by ELISA. With the truncated pRNA I-i′ 23–97, the mediation effect of pRNA in the interaction between gp16 and procapsids was reduced substantially (Figure 6). These results agree with previous findings indicating that deleted pRNA at the 5′/3′ end lost DNA-packaging activity, and the results suggest that the domain at the 5′/3′ paired ends plays a role in pRNA–gp16 interaction.

To further investigate the role of the $C_{18}C_{19}A_{20}$ bulge and to determine whether this bulge is essential for gp16–pRNA interaction, pRNA 7/GGU, which exhibited loss of DNA-packaging activity by insertion of additional UGG sequences to pair with and eliminate the CCA bulge,[64] was added to procapsids, and then loaded onto gp16 affinity column as described above. When procapsids in eluents were measured by ELISA after elution by 0.5 M NaCl, procapsids were significantly eluted in amounts as high as when

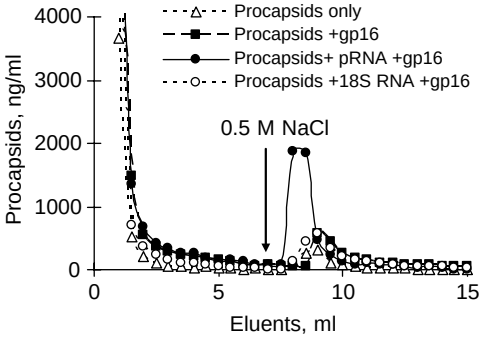

Figure 5. Specific binding of gp16 to the procapsid–pRNA complex. Mixtures of procapsid with pRNA (filledcircle) or with 18 S RNA (open circle) were applied to a gp16 affinity column and eluted by 0.5 M NaCl. The procapsid concentration of each fraction was measured by ELISA using phi29 procapsid-specific rabbit IgG. Procapsid alone was flowed through the affinity column with (filled triangle) or without (open triangle) gp16 to evaluate the non-specific interaction.

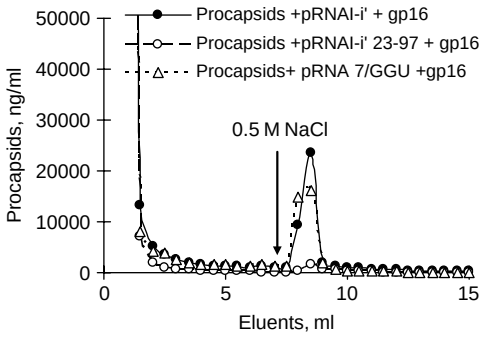

Figure 6. Demonstration of the role of the 5′/3′ paired ends of pRNA for gp16 binding. Procapsids were mixed with pRNA I-i′ (filled circle), pRNA I-i′$_{23\text{-}97}$ (a truncated pRNA containing nucleotides only from base 23–97, with base 1–22 and 98–120 deleted; open circle) and pRNA I-i′$_{7/GGU}$ (a pRNA with the $C_{19}C_{20}A_{21}$ bulge removed; open triangle), respectively, before application to the gp16 affinity column. The procapsid concentration after elution by 0.5 M NaCl was calculated through ELISA.

the wild-type pRNA was used (Figure 6). These results indicated that the CCA bulge-free pRNA 7/GGU could still mediate the interaction between procapsids and gp16. Therefore, the CCA bulge is not essential for gp16 binding, although it is indispensable for DNA packaging.

Discussion

To understand the role of gp16 during motor action, it is critical to determine whether gp16 interacts with DNA–gp3 or pRNA and whether any possible interaction is specific or non-specific. The question of specificity includes whether gp3 or specific signals of DNA sequences are needed for binding or interaction. If gp16 is needed only at the initiation of DNA packaging, for instance as a DNA loader,[65] then gp3 or a sequence-specific signal will be required for gp16–DNA–gp3 interaction. If, on the other hand, gp16 is not only needed for the initiation of DNA packaging, but also serves mainly as a processive factor during DNA translocation, then gp16 could also interact with DNA non-specifically. It has been reported that the large subunit of phage T4, gp17, binds to the portal protein of procapsid and packages concatemeric DNA, whereas gp16 of phage of T4 binds preferentially to dsDNA containing gene *16* sequences.[37]

The interaction of gp16 with DNA–gp3[66] and pRNA[49,58] was previously investigated, and it was found that gp16 did not bind pRNA[49] and bound DNA only in the presence of gp3.[66] However, our new results reveal that binding of DNA to gp16 is independent of the presence of gp3, since both DNA–gp3 and gp3-free DNA were retained with gp16 in the gp16 column (Figure 1). This finding was confirmed in a binding assay using the EcoRI restriction DNA fragments with and without gp3. All DNA fragments, the gp3-bound left and right-arm as well as internal B, D and E fragments, appeared on the electrophoretogram after elution without significant differences (Figure 2). It seems contradictory to the previous report that the sedimentation of both left and right-end restriction fragments of phi29 DNA with gp16 was dependent upon the presence of gp3 at the end of DNA.[66] However, these two different results might represent different mechanisms of interaction, since sucrose gradient sedimentation reveals only the gp3-induced conformational change of DNA. Sucrose gradient sedimentation might not be sensitive enough to show the binding of gp16 to DNA.

Furthermore, it was clear that the binding of gp16 to DNA did not need a specific target sequence, since non-phi29 genomic DNA can compete for binding to gp16 (Figure 3). As noted above, if the DNA-packaging motor requires gp16 only for its initiation, then gp16–DNA–gp3 interaction would need a sequence-specific signal at the left end, where packaging initiates. Our results suggest that gp16 might be a part of a processive factor during DNA packaging. However, it remains to be determined that the interaction between gp16 and gp3 at the end of DNA is necessary to initiate the packaging process.

pRNA, another key factor of the packaging motor system, contains two separate functional domains: the procapsid-binding domain is located at the central section of the molecule,[67,50,51] bases 23–97, and the DNA-translocation domain is found at the 5′/3′-paired ends.[68] The procapsid binding domain has been well-characterized and has been found to bind the connector,[47,51,56,67] while the exact role of the DNA-translocation domain has not yet been elucidated. Immobilized gp16 on an affinity column showed that gp16 interacted with pRNA with specificity. The eluent fractions had a significantly higher peak of [^{3}H]pRNA than that of [^{3}H]18 S RNA in the control experiment (Figure 4(a)). This result was confirmed *via* a filter binding assay with the aid of soluble gp16, although it has been reported that gp16 did not bind to purified pRNA on the filter binding assay.[69] When radioactively-labeled pRNA binds to protein, pRNA attaches to the glass fiber filter due to the retention of the protein on the filter. gp16 held [^{3}H]pRNA on the glass fiber filter, while BSA did not (Figure 4 (b)). The specificity of gp16 binding to pRNA is in contrast to the non-specificity of gp16 binding to DNA. This means that gp16 needs to be docked in the specific site on the packaging motor, and the results show that pRNA can be the specific site for gp16 binding. We also found that pRNA can mediate the interaction between gp16 and procapsids, since, in the presence of pRNA, procapsids showed gp16 binding activity (Figure 5). 18 S RNA could not support procapsids for gp16 binding, which means that pRNA binding to gp16 is highly specific and is critical to the formation of the motor complex.

Furthermore, this mediating effect of pRNA was decreased when the 5′/3′ end of the pRNA was truncated (Figure 6). On the contrary, the mediating effect of pRNA 7/GGU remained with an efficiency similar to that of wild-type pRNA (Figure 6). pRNA 7/GGU has exhibited loss of DNA-packaging activity by the insertion of an additional three bases, UGG, between the A_{99} and A_{100} to pair with and remove the $C_{18}C_{19}A_{20}$ bulge.[64] Therefore, it can be inferred that the 5′/3′ paired ends of pRNA might serve as a binding site to gp16, and that the gp16 recruited by the procapsid–pRNA complex forms a packaging motor which is ready to interact with genomic DNA and to translocate DNA. The data also suggest that the CCA bulge is not involved in gp16 interaction.

The AAA+ family (ATPases associated with a variety of cellular activities) is a huge group of proteins commonly found in many biological systems.[70–73] This group of proteins, for example the clamp loader subunits and the DNA replication proteins, forms a hexamer or pentamer, and many of them interact with DNA.[74–76] A clamp loader is required to load the ring-shaped clamps that tether

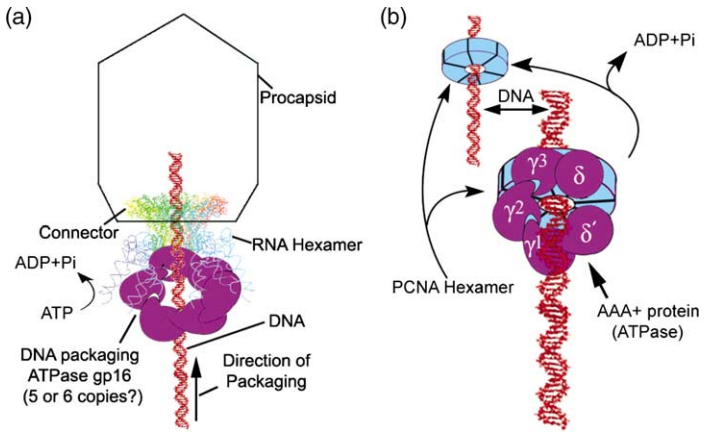

Figure 7. Proposed model of the phi29 DNA-packaging motor. (a) gp16 with unknown stoichiometry binds directly to the pRNA hexameric ring and packages genomic DNA–gp3 into the procapsid. (b) PCNA and clamp-loader complex. A clamp loader is required to load the ring-shaped clamps that tether replicative DNA polymerases onto DNA.

replicative DNA polymerases onto DNA. Other AAA+ proteins are also involved in the initiation of DNA replication. The crystal structures of many members of this family have been solved, including delta-prime[77] and the D2 domain of NSF.[78,79] Our results indicate that gp16 could be a hexameric or pentameric AAA+ protein which binds ATP and interacts with DNA, such as a pentameric clamp loader,[65,80,81] hexameric helicase[82–86] or T antigen.[87–89] In this model, gp16 binds pRNA to form an ATPase complex, which binds to the procapsid through pRNA–connnector interaction.[47] The complex driven by ATP hydrolysis helps generate torque to rotate the packaging motor to translocate genomic DNA like a bolt (Figure 7). Investigating the similarities between DNA-packaging motors and other hexameric DNA/RNA processing protein enzymes,[80–82,90–96] including DNA-helicases and the transcription termination factor Rho, will facilitate a better understanding of DNA replication and RNA transcription. Studies on DNA packaging could also reveal similarities to macromolecular transport across cell membranes, as is the case when mRNA and tRNA are transcribed in the nucleus and must pass through the nuclear membrane to reach the cytoplasm. pRNA dimers and trimers, and the rest of the motor as a whole, have been shown to have significant potential to be used as parts in biological nanotechnology.[7–9,97–99]

Materials and Methods

Expression and purification of His-tagged gp16

His-tagged gp16 was overexpressed in *E. coli* BL21 (DE3) by induction with isopropyl-β-D-thiogalacto-pyranoside (IPTG) followed by purification through one-step metal binding affinity chromatography as described.[59,60] Briefly, the gene coding for gp16 was cloned into plasmid pET-32, which contains a His-tag and S-tag coding sequence and a thioredoxin gene driven by a T7 promoter, and the plasmid was then transferred into *E. coli* BL21(DE3)/pLys for expression. The His-tagged gp16 was purified by one-step affinity chromatography using a His-tag column.[59,60]

Purification of phi29 DNA–gp3, gp3-free DNA, pRNA and procapsids

phi29 DNA–gp3,[100] procapsid[61,62] and pRNA[101] were prepared as described. The gp3-free DNA was prepared by treatment with 1 mg/ml of proteinase K for 1 h at 55 °C to remove the gp3. pRNA I-i′ 23–97[68] and pRNA I-i′ 7/GGU[64] were prepared as described.

Studies on the interaction of gp16 with phi29 DNA-gp3

A 1 ml syringe with a 23-gauge needle was used to pack 1 ml of His-Bind resin (Novagen), which was run under a gravity flow rate of 4 ml/h at 4 °C. After washing with 10 ml of water, the resin was charged with 10 ml of 50 mM $NiSO_4$, and equilibrated with 10 ml of His-binding buffer (20 mM Tris–HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, 15%(v/v) glycerol). Then 1 mg of the purified gp16 in 10 ml of His-binding buffer was loaded, followed by washing with 10 ml of His-binding buffer. The column was then re-equilibrated with 10 ml of nucleic acid binding buffer (15% glycerol, 1 mM ATP, 20 mM imidazole, 0.05%(v/v) β-mercaptoethanol). Then 1 μg of [^{3}H]phi29 DNA-gp3 or [^{3}H]phi29 gp3-free DNA was mixed with nucleic acid binding buffer to a final volume of 100 μl and then loaded to the column. The column was washed by nucleic acid binding buffer and was eluted with 1 M imidazole in nucleic acid binding buffer. The fractions were collected and analyzed by liquid scintillation counting.

Searching for specific signals on phi29 DNA for interaction with gp16

A total of 10 μg of phi29 gp3–DNA or phi29 gp3-free DNA was restricted by ten units of EcoRI for 1 h at 37 °C, producing five DNA fragments with different sizes (Figure 2(a)), as shown on a 0.8%(w/v) agarose gel (Figure 2(b)). The DNA fragments were resuspended in 100 μl of nucleic acid binding buffer and applied to the 1 ml affinity column containing gp16. After elution by 1 M imidazole in nucleic acid binding buffer, the collected fractions were concentrated by conventional ethanol precipitation and subjected to 0.8% agarose gel.

Competition assay to study the specificity of the interaction of gp16 with DNA

A total of 4 μg of unlabeled herring sperm DNA was mixed with 1 μg of [^{3}H]phi29 gp3-free DNA in 100 μl of

nucleic acid binding buffer and then loaded to a 1 ml gp16 affinity column as described above. After washing with nucleic acid binding buffer, the column was eluted with 1 M imidazole in nucleic acid binding buffer and the fractions were subjected to liquid scintillation counting.

Studies on the specificity of pRNA and gp16 interaction

A total of 50 pmol of [^{3}H]pRNA I-i′ in 100 μl of nucleic acid binding buffer was loaded onto a 1 ml affinity column charged with or without gp16 as described above. After washing with nucleic acid binding buffer, the column was eluted with 0.5 M NaCl in nucleic acid binding buffer. The collected fractions were analyzed by liquid scintillation counting. An equal amount of [^{3}H]18 S RNA was introduced as a control.

Determination of whether pRNA is needed for the interaction of gp16 with procapsid

The 1 ml of gp16 affinity column was prepared as described above. Procapsid–pRNA I-i′ complex[59] was diluted with nucleic acid binding buffer to 100 μl and loaded onto the column. After washing with nucleic acid binding buffer, the column was eluted by 0.5 M NaCl in nucleic acid binding buffer. The collected fractions were analyzed by ELISA with phi29 procapsid-specific rabbit IgG. Briefly, a 96 well microplate (Corning) was coated with 100 μl/well of each fraction diluted ten times by 50 mM sodium carbonate buffer (pH 9.5) and incubated overnight at 4 °C. Each well was blocked with 200 μl of 1% TPBSA (phosphate buffered saline (pH 8.0), 1%(w/v) bovine serum albumin, 0.05%(v/v) Tween-20). The microplate was treated with 100 μl/well of 1% TPBSA containing procapsid-specific rabbit IgG for 1 h. Then 100 μl/well of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was added, followed by 0.03%(v/v) peroxide and OPD as substrate for the color reaction, which was stopped by the addition of 100 μl/well of 0.5 M H_2SO_4. The microplate was analyzed on a microplate reader at a wavelength of 490 nm. 18 S RNA was used as a control.

Determination of the role of the 5′/3′ DNA-packaging domain of pRNA for gp16 interaction

The 50 pmol of pRNA I-i′ 23–97, a truncated pRNA with deletion of nucleotides 1–22 and 98–120, or pRNA I-i′ 7/GGU, a pRNA with flattening of the CCA bulge, was mixed with 20 μl of procapsids as described above. After dilution with nucleic acid binding buffer to 100 μl, the mixture was loaded onto a 1 ml gp16 affinity column as described above. The column was washed with nucleic acid binding buffer and eluted by 0.5 M NaCl in nucleic acid binding buffer. The procapsids in collected fractions were analyzed by ELISA as described above.

Filter binding assay for pRNA–gp16 interaction

The filter binding assay was performed as described.[49] Briefly, 1 pmol of [^{3}H]pRNA I-i′ in 1 μl of RNase-free water was mixed with various concentrations of gp16 in nucleic acid binding buffer to a final volume of 20 μl, followed by incubation for 10 min at ambient temperature. Glass fiber filter (Millipore) was soaked in nucleic acid binding buffer for 30 min. The mixture was filtered through the pre-soaked glass filter and washed with 2 ml of nucleic acid binding buffer per filter membrane. After drying in air, the membrane was subjected to liquid scintillation counting.

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