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Modulation of the Viral ATPase Activity by the Portal Protein Correlates with DNA Packaging Efficiency[*]

Leonor Oliveira[‡1], **Adriano O. Henriques**[§], **and Paulo Tavares**[‡]

From the [‡]*Unité de Virologie Moléculaire et Structurale, Unité Mixte de Recherche (UMR) CNRS 2472, UMR INRA 1157 and Institut Fédératif de Recherche 115, Bâtiment 14B, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France and the* [§]*Laboratory for Microbial Development, Instituto de Tecnologia Química e Biológica, Apartado 127, 2781-901 Oeiras, Portugal*

DNA packaging in tailed bacteriophages and herpesviruses requires assembly of a complex molecular machine at a specific vertex of a preformed procapsid. As in all these viruses, the DNA translocation motor of bacteriophage SPP1 is composed of the portal protein (gp6) that provides a tunnel for DNA entry into the procapsid and of the viral ATPase (gp1-gp2 complex) that fuels DNA translocation. Here we studied the cross-talk between the components of the motor to control its ATP consumption and DNA encapsidation. We showed that gp6 embedded in the procapsid structure stimulated more than 10-fold the gp2 ATPase activity. This stimulation, which was significantly higher than the one conferred by isolated gp6, depended on the presence of gp1. Mutations in different regions of gp6 abolished or decreased the gp6-induced stimulation of the ATPase. This effect on gp2 activity was observed both in the presence and in the absence of DNA and showed a strict correlation with the efficiency of DNA packaging into procapsids containing the mutant portals. Our results demonstrated that the portal protein has an active control over the viral ATPase activity that correlates with the performance of the DNA packaging motor.

The mechanism by which viral DNA is packaged into a preformed procapsid is a fascinating but still unsolved biological problem. Tailed phages and herpesviruses package DNA to densities as high as 500 mg/ml in the preformed procapsid structure (1–3). The DNA translocating machine that accomplishes this task is the most powerful biomolecular motor presently described (4, 5). Like many other molecular motors, this machine converts the chemical energy generated by ATP hydrolysis into mechanical movement of DNA. Intensive research over the past years enabled the identification and characterization of the components of the DNA translocating motor in several bacteriophages, as well as in herpesviruses (reviewed in Ref. 6). Despite some differences that may subsist between individual virus species, numerous biochemical and structural similarities have been found among the motor components, suggesting a common mechanism for DNA translocation. The motor is composed of an ATPase (terminase) and of the portal protein, a turbine-like oligomer localized at the unique vertex of the icosahedral capsid through which DNA movements occur (7–11). Packaging is initiated by docking of the DNA-terminase complex at the portal vertex. DNA is then translocated through the central tunnel of the oligomeric portal protein to be packed at high density inside the viral capsid. Translocation of the double helix is fueled by the terminase ATPase activity (12–16). Packaging is normally terminated by an endonucleolytic cleavage of the concatemeric DNA used as substrate for encapsidation by most phages (3). Previous work defined the components of the motor but elucidated neither how the DNA packaging motor assembles nor the role of the portal protein in DNA translocation.

The *Bacillus subtilis* bacteriophage SPP1 is a well established model system for viruses that package their DNA into procapsids with a portal (16, 17). The packaging reaction requires substrate DNA, ATP, the terminase gp1-gp2 complex, and procapsids with the portal protein gp6. The terminase small subunit gp1 provides specificity for binding to the *pac* sequence in the viral DNA concatemer (18, 19), whereas the large subunit gp2 has ATPase and endonuclease activities (20, 21). gp6, like portals from other phages, is a cyclical homo-oligomer (a 745-kDa 13-mer in solution and a 688-kDa 12-mer when incorporated in the phage structure) (22) with a central tunnel for DNA translocation. The purified protein modulates the ATPase activity of the terminase (21). At the end of genome encapsidation, gp6 is a central component of the sensor system that triggers headful cleavage of substrate DNA after a threshold amount of DNA is reached inside the capsid (8, 23). Single amino acid substitutions in gp6 can block the initiation of DNA packaging, its efficiency, or termination of the process that yields the ~45.9-kbp long mature viral chromosome (24, 25).

Here we describe the cross-talk between the SPP1 terminase and the portal protein. gp6 embedded in the procapsid structure caused a strong stimulation of the gp2 ATPase activity that depended on the presence of gp1. Mutations in different regions of the portal protein decreased or abolished this effect irrespective of the presence of DNA. Most interestingly, the capacity of procapsids carrying different gp6 forms to boost ATP hydrolysis by gp2 correlated with their efficiency to encapsidate DNA *in vitro*. Regulation of the viral ATPase activity by the portal protein in the assembled DNA translocation motor appeared essential for the mechanism of viral genome packaging.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Ultrapure acrylamide and isopropyl 1-thio-β-D-galactopyranoside were purchased from Euromedex. Agarose was from Bio-Rad. Syber Gold was from Molecular Probes. Protein molecular weight markers and DNA restriction enzymes were from New England Biolabs. Proteinase K was from Roche Applied Science. Lysozyme, sodium molybdate, DNase, and dextran were from Sigma. ATP was purchased from Roche Applied Science. [γ-^{32}P]ATP was from Amersham Biosciences.

Bacterial Strains, Bacteriophages, and Plasmids—SPP1 suppressor-sensitive mutants (SPP1*sus115*, *sus70*, and *sus70sus115*) and SPP1*sizX* were described previously (18, 23, 25). *B. subtilis* HA101B (*sup-3*) and YB886 (*sup^o*) were the permissive and nonpermissive strains used for SPP1 multiplication (18). Handling of bacterial and phage strains was as described previously (18, 23). The *Escherichia coli* strain used for gp6 overproduction was XL-1 Blue (Stratagene). *E. coli* strains used for production of the terminase subunits gp2 and gp1 were BL21(DE3) and BL21(DE3)(pLysS), respectively (26).

Plasmids pCB191 (27), pBT115 (18), and pREP4 (Qiagen) have been described. An Hpa-BclI fragment of SPP1 DNA carrying gene *6* (23) (coordinates 2267–3917 in the SPP1 sequence, GenBank^{TM} accession number X97918) (28) was cloned downstream of the inducible promoter $P_{N25/O}$ (29)[2] in a derivative of plasmid pHP13 (30). Expression was controlled by the LacI repressor coded by plasmid pGB3 in *E. coli* or by pEB104 in *B. subtilis*, respectively (31). Missense mutations in gene *6* were transferred from pFiFAcc (24) to the vector by subcloning PflMI-Asp718 (coordinates 3040–3244 of the SPP1 sequence) (mutations S279G, N290T, and E294K) or Asp718-PstI fragments (coordinates 3244–3917 of the SPP1 sequence; the PstI site is from the vector polylinker) (mutations T319A and E352G). The replacement strategy is as described (25). Gene *6* was then sequenced to confirm the presence of the mutation under analysis and to certify the absence of additional mutations. Cloning in this vector allowed us to control the expression of the gene *6* alleles and to increase significantly the level of production of gp6 relative to the original constructs derived from pFiF (25).

DNA Manipulations—Plasmid DNA was purified with a plasmid purification kit from Qiagen or by using the SDS-lysis method followed by purification on a cesium chloride-ethidium bromide gradient (32). Mature phage SPP1 DNA was extracted from phage particles by phenol/chloroform extraction followed by precipitation with isopropyl alcohol. DNA concentration was determined using molar extinction coefficient of 6500 $M^{-1}\cdot cm^{-1}$ at 260 nm. Analytical gel electrophoresis of SPP1 DNA, plasmid DNA, and restriction fragments was carried out in 0.8% (w/v) agarose/Tris borate/EDTA horizontal slab gels.

Analysis of gp6 Production—The production of gp6 in *B. subtilis* strains expressing different gene *6* alleles was determined by immunoblot analysis of crude extracts developed with an anti-gp6 antibody (24).

Production and Purification of SPP1 Procapsids Carrying Different gp6 Forms—Wild type SPP1 procapsids were produced by infection of the YB886 *B. subtilis* strain with SPP1*sus70*, which has a nonsense mutation in gene *1* that codes for gp1, to an input multiplicity of 5. Procapsids carrying different forms of gp6 (gp6$_{SizX}$ or other gp6 mutants that affect DNA packaging *in vivo*) were produced by infection of the *B. subtilis* strains bearing the respective gene *6* alleles with SPP1*sus70sus115*. This phage has nonsense mutations in both genes *1* and *6*. The gene *6* allele coded by the plasmid complements the phage deficiency in *trans*. Procapsids were purified on 10–30% glycerol gradients followed by an anionic exchange chromatography (16). The molecular mass estimated for the procapsids was about 1.9×10^7 Da. The purity of the procapsid preparations and the amount of gp6 incorporated in the procapsid structures were analyzed by SDS-PAGE and by Western blotting with an anti-gp6 antibody. Blots were developed using the ECL detection system (Amersham Biosciences). The quality of the different procapsid preparations was also checked by electron microscopy of negatively stained samples.

Protein Purification—gp6 was purified with some modifications to the method described previously (33). Briefly, the *E. coli* XL-1 overproducing strains bearing the gene *6* different alleles were concentrated in buffer G (50 mM Hepes, pH 7.6, 10 mM MgCl$_2$, 500 mM NaCl, 10% (v/v) glycerol) and lysed by passing through a French press, and the protein material was precipitated with ammonium sulfate (70% (w/v) saturation). The pellet was resuspended in buffer A (20 mM Hepes, pH 7.6, 10 mM MgCl$_2$, 50 mM NaCl) and dialyzed overnight against buffer A. The protein was purified by ionic exchange chromatography in a fast protein liquid chromatography system using a Resource Q column (Amersham Biosciences) eluted with a linear gradient from 50 to 500 mM NaCl in buffer A. gp6 was further purified by size exclusion chromatography in a Superose 6 column (Amersham Biosciences). The pure protein was dialyzed against buffer A, and aliquots were stored at −80 °C. gp1 and gp2 were purified as described previously (16). Protein concentration was determined by the Bradford method (34), using bovine serum albumin as a standard. gp6, gp1, and gp2 concentrations were expressed as moles of protein 13-mers (35), decamers (19), and monomers (20), respectively.

ATPase Activity Measurement—The ATPase standard reactions were incubated in 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM dithiothreitol, and 10 mM MgCl$_2$, in a final volume of 20 μl. Unless stated otherwise, the concentrations of ATP, gp2, gp1, and procapsids used were 1 mM and 200, 300, and 20 nM, respectively. The reactions were initiated by addition of the substrate after a preincubation of 5 min at 30 °C and were allowed to proceed for 15 min more at the same temperature. ATPase activity was determined by measuring the amount of phosphate set free upon hydrolysis as described previously (36). In brief, the reaction was terminated by the addition of 200 μl of 1.5 mM NaH$_2$PO$_4$ in 0.5 M H$_2$SO$_4$, 50 μl of molybdate reagent (200 mM Na$_2$MoO$_4$), and 500 μl of isobutyl alcohol. The mixture was shaken vigorously, and the phases were allowed to separate. After centrifugation for 5 min, radioactivity in the upper isobutyl alcohol layer and in the lower aqueous phase was measured in a liquid scintillation counter. The ATPase-specific

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[2] P. Tavares, unpublished results.

TABLE 1

In vivo characterization of gp6 mutations that affect DNA packaging

Amino acid substitution[a]	Titer[b]	gp6 levels[c]	gp6 incorporation in procapsids[d]	DNase protection[e]	Mature DNA size[e]	Phenotype	Classification[f]
	% of control						
SizX (control)	100	$+++++$	$+++++$	$+++++$	Normal		
S279G	2.9	$+++++$	$+++++$	$+$	$<$	Low packaging efficiency, smaller chromosome	Group II
N290T	0.5	$+++++$	$++++$	$-$		No packaging	Group I
E294K	0.6	$+++++$	$+++++$	$-$		No packaging	Group I
T319A	10	$+++++$	$+++++$	$+$	$<$	Low packaging efficiency, smaller chromosome	Group II
E352G	2.9	$+++++$	$+++++$	$+$	Normal	Low packaging efficiency	Group III

[a] The gp6 single amino acid substitutions are caused by missense mutations in gene *6* characterized in this work.
[b] The severity of the mutations was estimated in complementation assays by titration of SPP1*sus115* phages (gp6⁻) in strains bearing plasmids coding for gene *6* mutant alleles and estimated as percentage of the titer (plaque-forming units/ml) obtained with the strain coding for SizX. Values represent the average of three independent experiments.
[c] Accumulation of gp6 in the cell. The relative intensity of the gp6 signal in Western blot was expressed by comparison with the levels found for expression for the *sizX* allele.
[d] Amount of gp6 in purified procapsids. The procapsid samples applied for Western blot were normalized according to the levels of gp13 present in each procapsid preparation, as estimated from Coomassie Blue-stained gels. Relative signal intensity in Western blots is presented, considering $gp6_{SizX}$ as $+++++$.
[e] Characterization of viral DNA packaged. The presence of phage DNA and the size of the mature chromosome were determined by PFGE of total DNA from extracts of infected cells treated or not with DNase. The relative efficiency of packaging is estimated by comparison with the *sizX* allele, varying from $+++++$ (normal packaging efficiency) to $-$ (DNA not stably packaged). The size of the DNA packaged was expressed by comparison with the SPP1 *sizX* chromosome size, either (smaller) or normal (*sizX* chromosome size).
[f] Classification of the gp6 mutants according to the different *in vivo* DNA packaging phenotypes.

activity was determined by calculating the amount of P_i^* produced per min and expressed in terms of $\mu mol/min/mg$ gp2.

DNA Packaging Analysis in Vivo—*B. subtilis* strains bearing plasmids coding for the different gene *6* alleles under analysis were infected with SPP1*sus115* to an input multiplicity of 10. After 22 min the infected cells were lysed, treated with DNase, and analyzed by pulsed-field gel electrophoresis (PFGE),[3] as described (24). PFGE gels were stained with ethidium bromide or with SYBR Gold (Molecular Probes).

DNA Packaging Reaction in Vitro—DNA packaging reactions were performed *in vitro* with purified terminase proteins and procapsids in a DNase protection assay (16). Briefly, a standard 20-μl reaction mixture contained 1 mM ATP, 1 nM DNA, 10% dextran, 20 nM procapsids, and terminase proteins (200 nM gp2 and 300 nM gp1) in 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 10 mM MgCl₂. Reactions were incubated for 45 min at 30 °C, if not stated otherwise. DNase was then added at 20 μg/ml and incubation continued for 10 min at 37 °C. Reactions were stopped by addition of 50 mM EDTA and deproteinized. DNA was resolved by gel electrophoresis in 0.8% agarose gels and stained with Syber Gold (Molecular Probes) according to the manufacturer's instructions. The amount of DNA packaged was quantified by using ImageQuant software (Amersham Biosciences). DNA with known concentrations was loaded into the gel in parallel and was used as standard for quantification. The values used for quantification were within the linear range of the DNA calibration curve.

RESULTS

In Vivo Characterization of gp6 DNA Packaging Mutants—Random mutagenesis of gene *6* combined with a screening strategy enabled the previous identification of gp6 mutants that affect SPP1 DNA packaging (24, 25). A subset of these mutants was selected according to phenotype (data not shown). Three major groups typified by the amino acid substitutions compiled in Table 1 could be identified. All the mutant gp6 forms tested are normally produced and incorporated into SPP1 procapsids (Table 1). *B. subtilis* cells producing the different gene *6* alleles

[3] The abbreviation used is: PFGE, pulsed-field gel electrophoresis.

linked to mutation *sizX* (25) were infected with a phage defective in gp6 production (SPP1*sus115*), and DNA packaging *in vivo* was assessed by DNase protection assays followed by PFGE. The *sizX* mutation leads to packaging of SPP1 chromosomes with a size shorter than wild type but does not affect the efficiency of DNA encapsidation. Its linkage to gene *6* alleles is essential to distinguish between DNA packaged *in vivo* and background chromosomes with wild type size resulting from infecting phages used in complementation assays (25). A first set of mutations (group I typified by $gp6_{N290T}$ and $gp6_{E294K}$) blocked DNA packaging such that no protected DNA inside SPP1 capsids is detected after DNase digestion of extracts of infected cells (Table 1). Other gene *6* alleles were characterized by the protection of a small amount of packaged DNA appearing as a faint band in PFGE. They were classified as "low packaging efficiency" mutations. Within this second type of mutations we distinguish group II ($gp6_{S279G}$ and $gp6_{T319A}$) characterized by undersizing of the packaged DNA molecule and group III ($gp6_{E352G}$) whose DNA packaged molecules have the same size as observed for the *sizX* control allele (Table 1). An additional feature associated to mutation E352G (group III) is that *in vivo* studies suggested it causes a defect in DNA translocation (25). These mutants are thus likely candidates to arrest or affect different reactions during the DNA packaging process.

Effect of gp6 Mutations on the Stimulation of Terminase ATPase Activity by the Portal Protein—DNA packaging involves interaction of the DNA-associated terminase proteins with the prohead-associated portal protein (6). ATP hydrolysis by the terminase energizes the reaction. In SPP1, the terminase large subunit gp2 displays a low ATPase activity that is stimulated by the small subunit gp1. Purified gp6 (Fig. 1*A*), a 13-mer protein outside the context of the procapsid structure, also stimulates the activity of gp2, and mixing of the three proteins has a synergistic effect in ATP hydrolysis (20, 21) (Fig. 1*B*). We have thus investigated whether gp6 mutations that caused an absence of DNA packaging or a reduced packaging efficiency phenotype affected the stimulation of terminase (gp2 + gp1) ATPase activity. We first demonstrated that pure wild type gp6 and $gp6_{SizX}$ caused an identical stimulation of gp2 activity both in the presence and in the absence of gp1 (Fig. 2*B*). Because the

FIGURE 2. **Concentration dependence for purified procapsids and purified gp6 in the stimulation of the terminase ATPase activity.** The ATPase activity was determined in the presence of constant amounts of gp2 and gp1 (200 and 300 nM, respectively), and increasing concentrations of procapsids (*proc*) or of purified gp6. One representative experiment is shown.
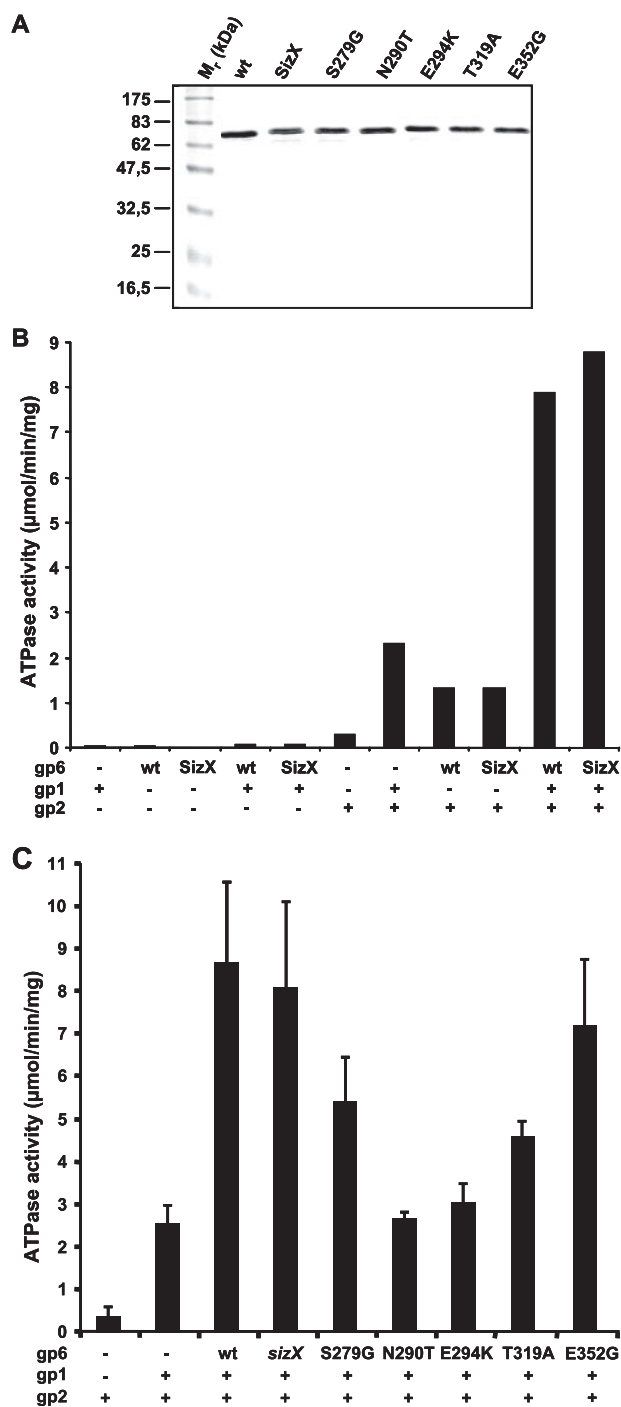

FIGURE 1. **ATPase activity of the terminase in the presence of wild type and mutant portal proteins.** *A*, SDS-PAGE analysis of the different gp6 proteins purified. *wt*, wild type. *B*, modulation of the terminase ATPase activity by wild type gp6 and gp6$_{SizX}$ (control). gp1 (300 nM decamers), gp2 (200 nM monomers), gp6 (80 nM 13-mers), and 1 mM ATP were mixed, and ATP hydrolysis was measured as described under "Experimental Procedures." One representative experiment is shown. *C*, effect of gp6 DNA packaging mutations on the stimulation of the terminase ATPase activity. The *bars* represent the average of five independent experiments with the standard deviations indicated.

chromatography and had the typical turbine-like appearance of gp6 cyclical oligomers in electron microscopy observations (data not shown). Fig. 1*C* shows the mean values and standard deviations of the specific ATPase activity of gp2 quantified in the presence of gp1 and of the mutant forms of gp6. We observed distinct effects of the mutations on the faculty of gp6 to boost the ATPase activity of terminase. A subgroup of mutations (N290T and E294K) caused a drastic effect. These mutants, in particular the gp6$_{N290T}$ protein, are unable to stimulate the ATPase activity of terminase. An intermediate rise of the ATPase activity of terminase was observed for another subset of gp6 mutants (S279G and T319A). Finally, the E352G substitution affects only slightly the ability of gp6 to enhance terminase ATPase activity (Fig. 1*C*). This screening defined three classes of mutants according to their capacity to stimulate ATP hydrolysis by the terminase. They were typified by the N290T, T319A, and E352G amino acid substitutions, which we decided to characterize in further detail.

*Modulation of the Terminase ATPase Activity by Procapsids—*The active portal protein that participates in DNA packaging is a 12-mer structure embedded in the capsid lattice defining a specialized DNA translocating vertex (22). To get further insight on the link between stimulation of ATPase activity and DNA packaging, it was thus fundamental to analyze the effect of procapsids with a portal structure in ATP hydrolysis by the terminase. ATPase reactions were carried out with fixed concentrations of gp1 and gp2 and increasing amounts of procapsids. The effect of procapsid addition on the ATPase activity of terminase was compared with the one obtained with purified gp6 in dose-dependent curves (Fig. 2). Interestingly, we found a dramatic difference in the capacity of procapsids to stimulate the specific ATPase activity of gp2 in the presence of gp1, relatively to pure gp6. The ATPase activity is noticeably higher in the presence of procapsids containing gp6$_{SizX}$ (more than 10-fold increase relative to gp1 + gp2) than in the presence of purified gp6$_{SizX}$ (about 3–4-fold increase) (Fig. 2). An enhancement of ATP hydrolysis by the terminase can already be

mutant proteins characterized in this work carry the mutation *sizX*, we used gp6$_{SizX}$ as control in all subsequent experiments.

The five selected proteins bearing single amino acid substitutions that specifically block DNA packaging were purified (Fig. 1*A*). They were shown to be oligomeric by size exclusion
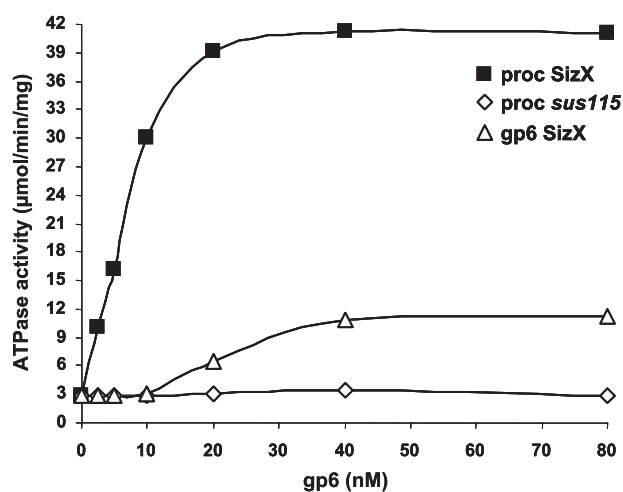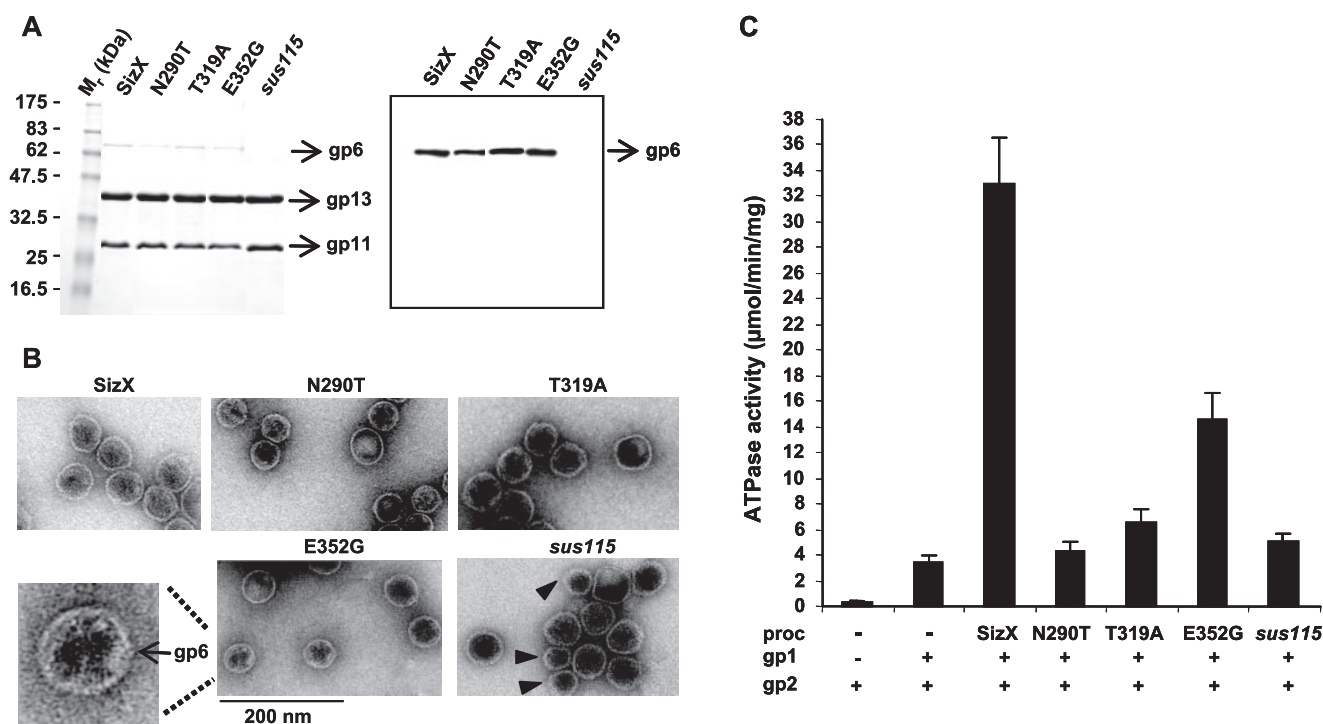
FIGURE 3. **Effect of gp6 DNA packaging mutations on the stimulation of the terminase ATPase activity by procapsids.** *A,* purification status of the different procapsid preparations analyzed by SDS-PAGE (*left panel*). The position of gp6, gp11, and gp13 is shown by *arrows*. The amount of gp6 present was also checked by Western blot analysis of the procapsid preparations with an anti-gp6 antibody (*right panel*). *B,* electron microscopy analysis of negatively stained preparations of purified procapsids. The *arrow* shows gp6 present at the portal vertex, and the *arrowheads* show smaller size procapsids present in the *sus115* (gp6⁻) procapsid preparation. *C,* ATPase activity of terminase in the presence of procapsids carrying different gp6 forms. The graphic shows the mean and S.D. of the results obtained for at least five independent experiments.

observed at low procapsid concentrations (2.5 nM), in contrast to pure gp6. The ATPase activity increased in a dose-dependent manner until around 20 nM procapsids, with 50% of the maximal stimulation reached with a procapsid concentration of 7.5 nM (Fig. 2). At concentrations above 20 nM, no further increase in ATPase activity is observed, suggesting that saturation is reached for the terminase concentrations used in these experiments. This striking stimulation is strictly dependent on the presence of gp6 in the procapsid, as no effect is seen with procapsids lacking gp6 obtained from infections with SPP1*sus115* (gp6⁻) (Fig. 2). In the absence of gp1, procapsids also increased the ATPase activity of gp2, although the effect was less pronounced (about 4- and 100-fold increase in the gp2-specific ATPase activity with or without gp1, respectively) (data not shown). As also observed with purified gp6, procapsids themselves showed no ATPase activity (not shown).

Effect of gp6 Mutations on the Procapsid-induced Stimulation of Terminase ATPase Activity—We then investigated whether procapsids carrying mutant gp6 forms affected the terminase ATPase activity in the same manner as observed for the purified mutant proteins. The protein composition and the purification level of the procapsid preparations were verified by SDS-PAGE. All the samples contain the three essential proteins of the procapsid, *i.e.* the major head protein gp13, the scaffolding protein gp11, and the portal protein gp6, with the exception of the gp6⁻ procapsids which, as expected, do not have portal protein (Fig. 3*A*). The integrity and quality of the procapsids were also confirmed by electron microscopy of negatively stained samples (Fig. 3*B*). Homogeneous populations of roundly shaped procap-

sid structures are observed in all samples with the exception of procapsids lacking gp6, where particles with two different sizes are seen. This is a typical feature of SPP1 gp6⁻ procapsids (37).

The effect of gp6 mutations on procapsid modulation of the terminase ATPase activity is shown in Fig. 3*C*. Procapsids carrying portals with the substitutions N290T and T319A have an impact on gp2 activity comparable with the one found for the corresponding isolated portals; no significant stimulation of the ATPase activity is seen with procapsids carrying gp6$_{N290T}$, whereas a modest 2-fold increase is detected when gp6$_{T319A}$ is present. Procapsids containing gp6$_{E352G}$ lead to a stronger increase in the ATPase activity of terminase (about 4-fold) compared with those carrying gp6$_{T319A}$. This stimulation is, however, lower than the one induced by control procapsids (about 10-fold) (Fig. 3*C*). ATPase assays were performed using different concentrations of mutant procapsids (ranging from 5 to 40 nM). The relative stimulation of ATPase activity by procapsids carrying different gp6 forms was essentially identical in this concentration range. The results shown were obtained with a concentration of 20 nM procapsids that leads to maximal stimulation of ATPase activity at the concentrations of gp1 and gp2 used throughout this work (Fig. 2; results not shown).

Stimulation of ATPase Activity by Procapsids in the Presence of DNA—We have then determined the effect of procapsids on the ATPase activity when DNA is present to mimic the conditions in which genome packaging takes place. The packaging substrate was plasmid pBT115 linearized by digestion with PstI (lpBT) (16). We first analyzed the effect of DNA on the ATPase activity of gp2 in the presence or in the absence of gp1, of pro-
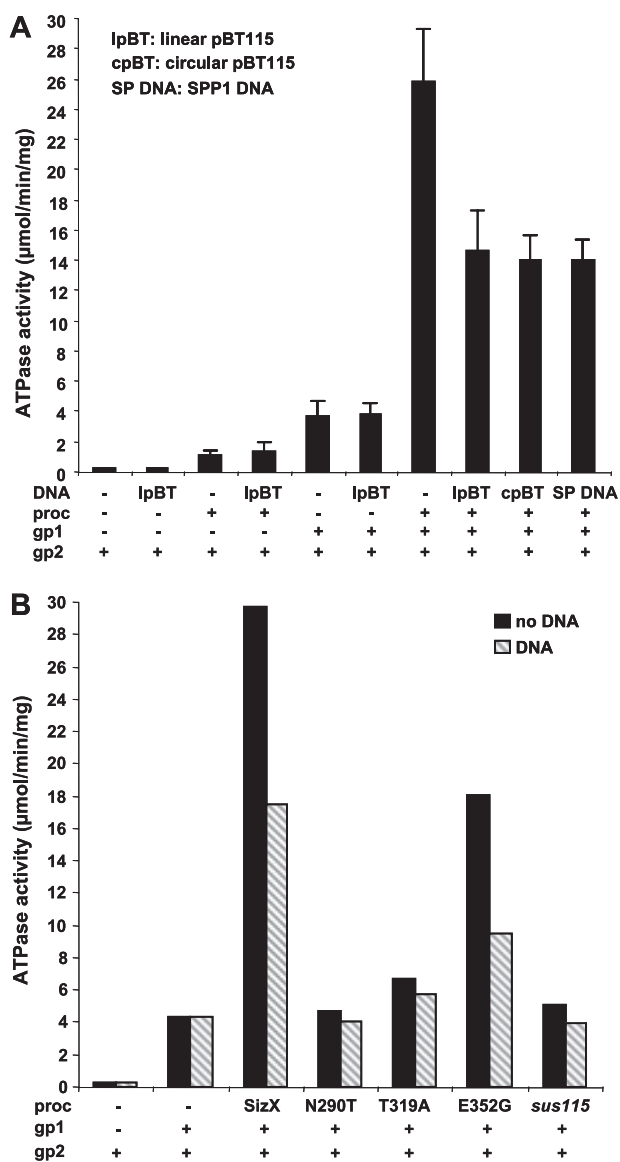
FIGURE 4. **Effect of DNA on the procapsid modulation of terminase ATPase activity.** *A*, effect of different DNA substrates on the stimulation of the terminase ATPase activity by procapsids. The lpBT and cpBT DNAs are the linear and circular forms of plasmid pBT115 (3.7 kbp), respectively. The lpBT substrate was prepared by digestion of pBT115 DNA with PstI. *SP DNA*, mature SPP1 DNA (45.9 kbp). All DNAs were added to a final concentration of 1 nM. *B*, effect of gp6 mutations in procapsid stimulation of terminase ATPase activity. Experiments were carried out in the presence or absence of 1 nM lpBT DNA. A representative experiment under the conditions used in Fig. 3*C* is shown.

which was packaged with the same efficiency as mature SPP1 DNA. The effect of circular pBT115 plasmid DNA (cpBT) and of mature SPP1 DNA (SP DNA) was thus compared with the results obtained with lpBT. All three substrates had an identical effect on the ATPase activity (Fig. 4*A*). Because cpBT was not a substrate for DNA packaging, the reduction in ATPase activity of gp2 caused by DNA in the presence of gp1 and procapsids could not be correlated with the process of DNA translocation. The reduction observed was less pronounced when increasing amounts of gp1 were added to the assay; 1.9-, 1.3-, and 1.2-fold reductions in ATPase stimulation were observed with gp1 concentrations of 300, 600, and 900 nM, respectively (data not shown). This observation suggested that the reduced procapsid-mediated stimulation of gp2 ATPase activity resulted from titration of the basic protein gp1 by DNA.

It is important to stress that although the overall effect of procapsid addition was reduced in the presence of DNA, the gp2 ATPase activity in reaction mixtures containing procapsids, gp1, and DNA was still significantly higher than the one observed in the presence of gp1 + gp2 or of gp1 + gp2 and purified gp6. Moreover, despite the smaller magnitude of the stimulation of ATP hydrolysis induced by procapsids in the presence of DNA, we observe similar relative effects dependent on the portal protein form present in the procapsid when compared with experiments carried out in the absence of DNA (Figs. 3*C* and 4*B*). Altogether, the experiments with portals embedded in the procapsid either in the absence (Fig. 3*C*) or in the presence of DNA (Fig. 4*B*) clearly demonstrated that the three gp6 mutations N290T, T319A, and E352G affected stimulation of the terminase ATPase activity to different levels. Mutation N290T caused the most severe reduction of ATP hydrolysis, followed by the T319A substitution, whereas E352G reduced by 50% the stimulation of gp2 activity. These effects correlated with the severity of their DNA packaging phenotypes *in vivo* (Table 1).

DNA Packaging into Procapsids Containing Mutant Portals—ATP hydrolysis energizes viral DNA packaging. We have thus investigated whether the different ability of procapsids containing mutant portal forms to stimulate the gp2 ATPase activity correlated with the DNA packaging efficiency of those procapsids. DNA packaging reactions were carried out *in vitro* using purified gp1, gp2, procapsids, and lpBT DNA. Packaged DNA was assayed by a DNase protection assay (16). This defined system enabled the direct quantification of DNA accumulation inside procapsids independently of additional factors required for completion of the viral particle assembly. Procapsids carrying gp6 wild type and gp6_SizX package DNA with the same efficiency (Fig. 5*A*, compare *lanes 2* and *3*). Quantification of the amount of DNA protected from DNase digestion revealed that 50 and 58% of the total input DNA was encapsidated, respectively.

The behavior of procapsids with gp6 containing mutations N290T, T319A, and E352G in DNA packaging is shown in Fig. 5*B*. DNA encapsidated in the different procapsids was quantified and expressed as percentage of control procapsids (Fig. 5*C*). Reproducible results were obtained with different preparations of procapsids containing the same gp6 form. All three mutations in gp6 caused a significant reduction of DNA packaging. The most severe effect is associated with gp6_N290T, with

capsids, and in the simultaneous presence of gp1 and procapsids. DNA addition did not cause any change in gp2 activity, either in the presence or in the absence of gp1 (Fig. 4*A*). Similarly, it did not significantly affect the level of stimulation induced by procapsids in ATP hydrolysis catalyzed by gp2, in the absence of gp1. However, a 2-fold decrease in ATPase activity was associated with the presence of lpBT in reactions containing gp1, gp2, and procapsids. To determine whether this reduction could be due to a regulatory effect related to the DNA translocation reaction, we tested the effect of DNA substrates with different properties. Previously, we have shown that circular plasmid DNA could not be packaged by the defined SPP1 *in vitro* packaging system, contrary to linear plasmid DNA,

FIGURE 6. **DNA packaging kinetics for procapsids carrying the gp6$_{SizX}$ (control), gp6$_{T319A}$, or gp6$_{E352G}$.** Conditions were as described under "Experimental Procedures," except that the packaging reactions were stopped at the indicated time points. The substrate for packaging was lpBT. *A,* DNase protection assay for different time points. *B,* quantification of packaged DNA with control and mutant procapsids at the different times. Packaging is expressed as nanograms of DNA protected from DNase quantified as described under "Experimental Procedures."
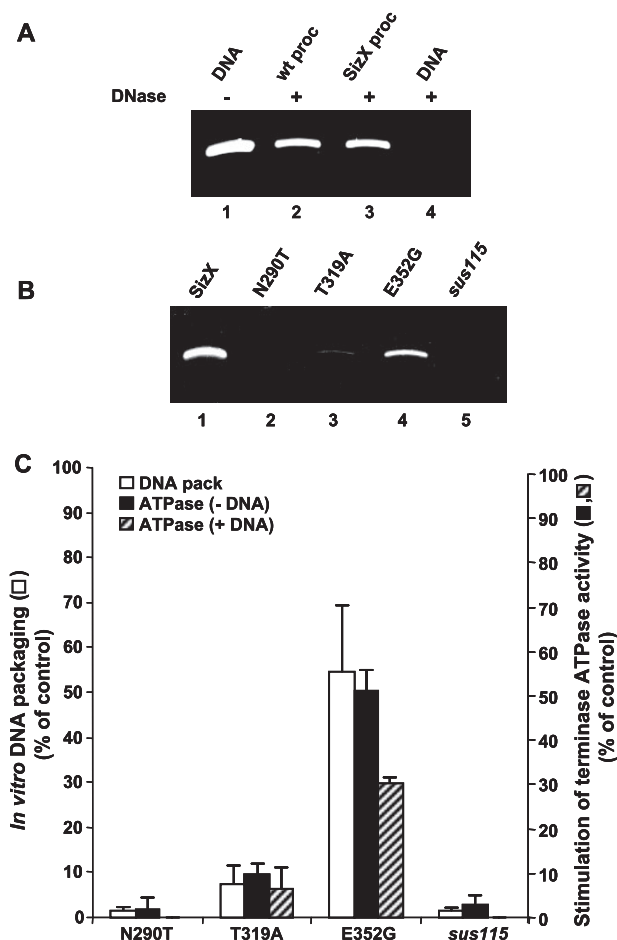
FIGURE 5. **DNA packaging in vitro into SPP1 procapsids carrying different gp6 proteins.** DNA packaging was assayed by a DNase protection assay in packaging reactions carried out with purified components for 45 min. Plasmid pBT115 linearized with PstI (1 nm) was used as substrate. *A,* packaging efficiency of SPP1 procapsids carrying wild type gp6 or gp6$_{SizX}$. *B,* effect of gp6 mutations in the DNA packaging reaction *in vitro*. *C,* quantification of the DNA packaging efficiency of procapsids carrying different gp6 forms (*open bars*) and of the stimulation of the terminase ATPase activity in the absence (*filled bars*) or in the presence (*bars* with *dashed filling*) of DNA and of the mutant procapsids (Fig. 4*B*). The amount of DNA protected is expressed in terms of percentage of the control procapsids with gp6$_{SizX}$. The graphic shows the mean and S.D. of the results obtained for at least four independent experiments. The stimulation of the terminase ATPase activity by procapsids was calculated by subtraction of the value obtained with terminase alone (gp1 + gp2). The results are expressed in terms of percentage of the control and represent the mean and S.D. obtained for three independent experiments.

DNA protection being barely or not at all detected, comparable with what is found for procapsids lacking gp6 (Fig. 5, *B* and *C*). A low efficiency of DNA packaging (7.2 ± 4.1% of control) is observed for the T319A substitution. Procapsids with gp6$_{E352G}$ package DNA *in vitro* more efficiently, although the amount of DNA protected is significantly inferior to the one obtained with the control (54.5 ± 14.6% of control). These results show a striking correlation between the effect of gp6 mutations in DNA packaging and the ability to stimulate the gp2 ATPase activity (Fig. 5*C*, *open* and *filled bars,* respectively).

Kinetics of DNA Packaging in Vitro—To examine further the DNA packaging efficiency of procapsids carrying control gp6, gp6$_{T319A}$, or gp6$_{E352G}$, we followed the kinetics of DNA encapsidation (Fig. 6). DNA packaging was observed after 2 min of incubation for all the experiments performed with control pro-
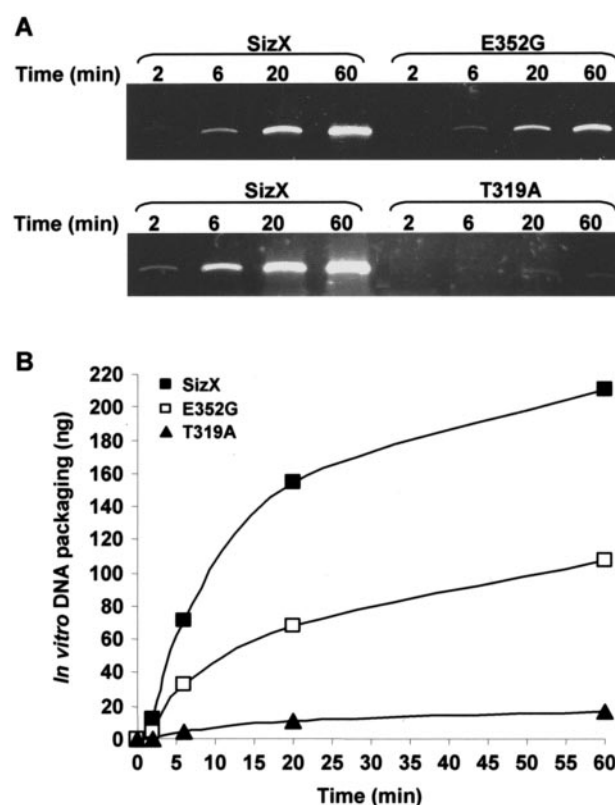
capsids. In most of the experiments DNA protection from DNase could also be detected with procapsids carrying gp6$_{E352G}$ within a 2-min packaging reaction, although to a lesser extent (around 40% of control). The amount of DNA packaged rises significantly from 2 to 20 min, and a smaller increase is seen at 60 min of the reaction (Fig. 6). The DNA packaging efficiency of procapsids with gp6$_{E352G}$ remained around 40–50% of control procapsids. As concerns the gp6$_{T319A}$ mutant, DNA packaging is only detected after 6 min and increases only slightly after that time (from 5.6 to 7.8% of the control at 6 and 60 min, respectively) (Fig. 6), thus confirming the low *in vitro* DNA packaging efficiency phenotype associated with this mutation.

DISCUSSION

The DNA translocation machinery that assembles at the specialized portal vertex of double-stranded DNA-tailed phages and herpesviruses packages DNA against a steep concentration gradient (4). Packaging of DNA into bacteriophage SPP1 procapsids was reproduced *in vitro* at high yield and characterized using purified components (DNA, the terminase gp1-gp2 complex, and procapsids with a portal structure) (16). These experiments defined the complete inventory of components of the SPP1 packaging motor. However, as in other viruses, the interactions between components of the motor and their concerted
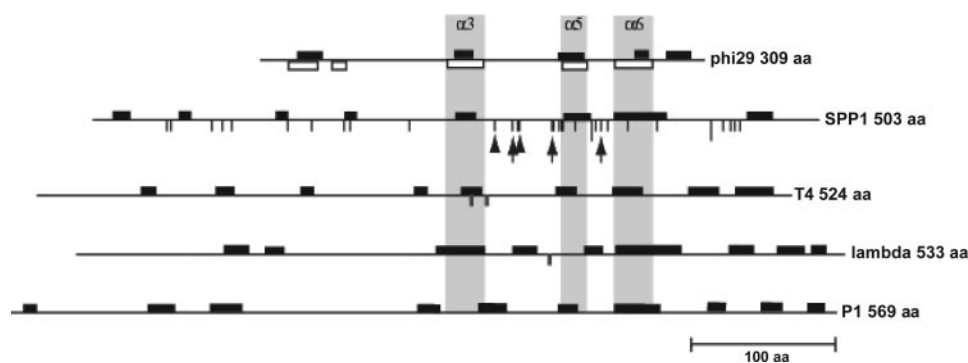
FIGURE 7. **Alignment of portal proteins through the α-helix elements on their secondary structure.** *White boxes* show the α-helices of the φ29 portal protein gp10 (9), and the *black boxes* represent predicted α-helices using the consensus secondary structure prediction software. Only α-helices with more than 9 amino acids are represented. The secondary structure predictions were aligned through the beginning of the putative helix 6 that was defined according to its long size and distance to putative helices 3 and 5. Note that the quality of the alignment according to these constraints is best for the portals of phages φ29, SPP1, and T4. The position of helices 3, 5, and 6 of the φ29 portal protein crystallographic structure is shown by *shading* in the alignment. *Vertical lines* indicate the position of mutations that impair DNA packaging (14, 15, 24). *Arrowheads* and *arrows* show the position of mutations in the portal protein of SPP1 that were selected and then subselected for the current study, respectively. *aa,* amino acid.

action to convert the energy of ATP hydrolysis into mechanical translocation of DNA remain to be established. Here, we characterized the control that the portal protein exerts on the viral ATPase activity and its impact in DNA packaging.

The first major finding stemming from our study was that the portal protein gp6 embedded in the procapsid structure converts gp2 into a powerful ATPase in the presence of gp1. This procapsid-induced stimulation is dose-dependent and is strictly dependent on the presence of a portal protein in the procapsid (Fig. 2). Procapsids with gp6 cause a much stronger stimulation of ATP hydrolysis than purified gp6 for identical concentrations of ATPase. Boosting of the gp2 ATPase activity (~100-fold relative to isolated gp2) is dependent on the presence of gp1 (Fig. 4A), the other essential component of the DNA packaging motor. This result demonstrated that discrimination between dodecameric portals in procapsids, which are the interaction partners for productive packaging reactions, and isolated 13-mer portals (38) was achieved by the viral terminase complex. It also revealed that interaction between all the proteins of the functional DNA translocation motor was necessary and synergistic to control the activity of its energy source.

The significant increase of ATP hydrolysis by the terminase upon assembly of the DNA packaging machinery is required to reach the turnover rate compatible with packaging of ≥300 bp·s^{-1} (16, 21) if we assume that SPP1 packages 2 bp of DNA per ATP hydrolyzed, as determined for phages φ29 (5, 12) and T3 (13). The strong boost of gp2 activity occurred in the absence and in presence of double-stranded DNA. A 1.2–2-fold lower stimulation observed in the latter case was attributed to binding of gp1 to DNA, preventing its association to the packaging motor (see "Results"). The presence of viral DNA has different effects on the hydrolysis of ATP by the terminase *in vitro,* depending on the bacteriophage species. The ATPase is DNA-dependent in bacteriophages such as T3 (39) and φ29 (12), whereas DNA does not affect ATP hydrolysis by the terminase in bacteriophage T4 (40). In bacteriophage λ a 2.6-fold increase in ATPase activity is observed in reactions initiated by addition of procapsids to the terminase-DNA complex (41).

Assembly of the SPP1 DNA packaging motor with high ATPase activity in absence of substrate DNA is not an economical strategy. However, the intimate association between SPP1 DNA and the terminase in extracts of infected cells suggests that *in vivo* a complex between these two viral components is formed first (38). The complex would then dock in the procapsid portal vertex to assemble the motor with concomitant initiation of DNA translocation.

The second major output of this work was the correlation between the level of stimulation of ATPase activity by the portal protein and the packaging efficiency of the DNA translocating motor. A set of single amino acid substitutions in the SPP1 portal protein presenting different DNA packaging phenotypes *in vivo* were identified (24, 25) (Table 1). Characterization of mutant portals representative of each type of phenotype demonstrated clear differences on their capacity to stimulate the terminase ATPase activity. gp6$_{N290T}$ and gp6$_{E294K}$ were unable to stimulate the ATPase, whereas other gp6 amino acid substitutions (S279G and T319A) caused a significant but less drastic reduction. The less severe mutation was the E352G substitution (Fig. 3C). Importantly, similar relative effects were observed when the ATPase measurements were performed with purified procapsids carrying the different gp6 proteins. This holds true both for experiments performed in the absence of DNA or with DNA present in the reaction mixture, under conditions where DNA packaging is expected to occur (Fig. 4B).

The availability of portal mutants that stimulate differently the viral ATPase activity provided an experimental framework to assess whether differences in the motor ATP hydrolysis rate had an impact on its DNA packaging performance. An evident correlation was observed between the two activities (Figs. 4B and 5), providing a biochemical basis to interpret the phenotypes associated to the gp6 mutations (Table 1). N290T mutant completely failed to stimulate ATP hydrolysis, and no significant DNA packaging could be observed in the *in vitro* experiments. The finding that this mutant failed to package DNA *in vitro* is in close agreement with the drastic reduction of titer in complementation assays and the total absence of DNA packaging seen by *in vivo* analysis (Table 1).

Procapsids carrying gp6 with the T319A substitution displayed a low *in vitro* DNA packaging capacity. DNA packaging was detected later than with control procapsids or procapsids carrying gp6$_{E352G}$, and the amount of DNA encapsidated stabilized at a low level after a 60-min reaction (about 10% of control). This might be a consequence of the presence of a very low number of procapsids that are competent for DNA packaging. However, the inefficient ATP hydrolysis by the packaging motor containing gp6$_{T319A}$ rather suggested that the low efficiency of packaging was because of a low speed or to a low processivity of DNA translocation. The *in vivo* analyses revealed that mutation T319A, besides leading to a

reduced packaging efficiency phenotype, leads to undersizing of the packaged DNA (24) (Table 1). This means that a low number of DNA-filled capsids are assembled during an infection cycle and that the headful sensor system triggers cleavage of the phage DNA before the wild type density of DNA is reached inside the capsid. The low stimulation of the ATPase activity by T319A procapsids and the low efficiency of *in vitro* packaging found in our experiments, combined with the *in vivo* data, led us to propose that a less effective DNA packaging machine caused a slowdown of DNA translocation and triggered an early signal for the endonucleolytic cleavage that terminates DNA encapsidation.

The E352G mutation caused a 50% reduction of DNA packaging *in vitro* (Fig. 5), which reproduces the effect of this amino acid substitution in the stimulation of ATPase activity (Fig. 4*B*). Studies *in vivo* suggested that capsids partially filled with DNA accumulate when this protein is present in procapsids (25). This is most consistent with a deficiency in the ability to complete DNA translocation than with a failure to initiate packaging. Next, and most important, although DNA-packaged molecules with normal size were detected *in vivo*, the amount of DNA packaged was low, and a reduced complementation level (about 3% of control) was found for this mutation (25) (Table 1). The *in vitro* and *in vivo* data are thus compelling to state that the E352G substitution strongly affects completion of the viral DNA packaging cycle. The less active ATPase might not be compatible with DNA translocation at late stages of the process when the DNA packaging motor has to exert huge force during DNA translocation (of around 57 piconewtons as reported with ϕ29 bacteriophage) (4), particularly when the internal pressure increases in the later stages of packaging. It can also be envisaged that the mutation affected the final steps of DNA encapsidation, such as stabilization of packaged DNA or/and concatemer cutting.

Our results do not allow us to define how the mutations in gp6 affect its cross-talk with gp2. They can either impair the protein-protein interaction and/or communication between gp2 and gp6 in the assembled packaging motor. The difficulty to assay for the physical interaction between the components of the motor during its transient assembly to package DNA did not allow us to address this question directly (21).[4] The drastic effect of substitution N290T suggests that it may abolish formation of the portal-terminase complex. However, the phenotypes of mutations T319A and E352G reveal that DNA packaging occurs, although inefficiently. This requires assembly of the packaging motor and processive translocation of DNA. Furthermore, mutation T319A affects the size of encapsidated DNA *in vivo*, whereas E352G has a defect to achieve complete DNA packaging (see above). Both phenotypes argue for inefficient cross-talk between gp2 and gp6 during DNA translocation. It remains to be established if such a defect adds to a weaker physical interaction between the motor components.

The crystallographic structure of the portal protein from bacteriophage ϕ29 revealed the presence of four long helices in the structure. Three of these are roughly parallel to the portal central tunnel (α1, α3, and α5), although helix α6 is oblique relative to the other long helices (9). Reconstruction of the por-

tal proteins of bacteriophages SPP1 and T7 based on cryo-electron microscopy data suggests that an identical pattern of helices in found on those proteins (11, 42). Furthermore, secondary structure predictions revealed the presence of putative long helices in numerous portal proteins. A pattern that resembled the α3-α5-α6 organization of gp10 from ϕ29 was found for several of those proteins. Their alignment is schematized in Fig. 7. This tentative structural assignment showed that the majority of amino acid substitutions that block DNA packaging in SPP1 mapped within the putative α3-α5-α6 region. Mutations in the portals of bacteriophages T4 (15) and λ (14) that affect the portal-terminase interaction are also localized in the same region (Fig. 7). The positions of residues Asn-290 and Thr-319 in the secondary structure alignment suggest that they would be localized at the bottom of the portal stem, the region expected to interact with the viral ATPase during DNA packaging. In contrast, Glu-352 would be localized between α5 and α6, a region in the center of the portal protein exposed to its internal tunnel (9). The recently determined crystallographic structure of gp6 showed that this tentative topology of residues is correct,[5] demonstrating that the terminase-portal protein cross-talk involves different regions of the portal structure. Interestingly, the position of mutation E352G suggests that it affects DNA translocation through the portal tunnel. This is compatible with an effect on the communication between the portal and the terminase rather than on the physical binding between these proteins.

Concluding Remarks—The results described in this paper strongly suggested that the portal protein plays an essential role to control ATP hydrolysis by the terminase to levels compatible with viral DNA translocation. The dramatic stimulation of the terminase ATPase activity induced by the portal protein in the procapsid translocating vertex is in harmony with the concept that the weak ATPase is changed into a translocating ATPase after gathering together of the packaging machine. In light of the tight relationship observed between the changes in the terminase ATPase activity found with the gp6 mutants and DNA translocation directly measured in the DNase protection assay, it is reasonable to propose that the portal-induced enhancement of ATP hydrolysis by the terminase is liable to DNA translocation. The challenge is now set to define how the intricate cross-talk between the two terminase subunits and procapsid-embedded portal protein transduces the chemical energy of ATP hydrolysis into mechanical translocation of DNA.

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Modulation of the Viral ATPase Activity by the Portal Protein Correlates with DNA Packaging Efficiency

Leonor Oliveira, Adriano O. Henriques and Paulo Tavares

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