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The portal protein plays essential roles at different steps of the SPP1 DNA packaging process

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Abstract

A large number of viruses use a specialized portal for entry of DNA to the viral capsid and for its polarized exit at the beginning of infection. These families of viruses assemble an icosahedral procapsid containing a portal protein oligomer in one of its 12 vertices. The viral ATPase (terminase) interacts with the portal vertex to form a powerful molecular motor that translocates DNA to the procapsid interior against a steep concentration gradient. The portal protein is an essential component of this DNA packaging machine. Characterization of single amino acid substitutions in the portal protein gp6 of bacteriophage SPP1 that block DNA packaging identified sequential steps in the packaging mechanism that require its action. Gp6 is essential at early steps of DNA packaging and for DNA translocation to the capsid interior, it affects the efficiency of DNA packaging, it is a central component of the headful sensor that determines the size of the packaged DNA molecule, and is essential for closure of the portal pore by the head completion proteins to prevent exit of the DNA encapsidated. Functional regions of gp6 necessary at each step are identified within its primary structure. The similarity between the architecture of portal oligomers and between the DNA packaging strategies of viruses using portals strongly suggests that the portal protein plays the same roles in a large number of viruses.

Keywords: Portal protein; DNA packaging; Molecular motor; Capsid expansion; Bacteriophage

Introduction

The viral DNA packaging mechanism is one of the most intriguing processes of viral assembly pathways. Double-stranded DNA (dsDNA) bacteriophages and herpes viruses package their DNA into a pre-assembled procapsid (Casjens and Hendrix, 1988; Homa and Brown, 1997; Valpuesta and Carrascosa, 1994). Procapsids are formed by a core of scaffolding protein surrounded by the major capsid protein arranged in an icosahedral lattice. The portal protein is located asymmetrically at 1 of the 12 vertices of the structure (Casjens and Hendrix, 1988; Valpuesta and Carrascosa, 1994). A DNA concatemer is the most frequent substrate for viral chromosome packaging (Black, 1989). In the majority of the phage systems, viral encoded proteins (terminase) bind to the DNA molecule in a specific sequence (*cos* or *pac*) and cleave it. The complex terminase–DNA then interacts with the portal vertex and DNA is translocated unidirectionally to the interior of the procapsid. Scaffold release from the procapsid interior takes place during DNA encapsidation, creating the necessary space to accommodate the viral chromosome. During DNA packaging, the capsid lattice undergoes a major conformational change (expansion) acquiring a more angular polyhedral shape (Hendrix and Duda, 1998). In some phages, capsid decoration proteins bind to the expanded capsid lattice (Casjens and Hendrix, 1988). The DNA packaging cycle is terminated by an endonucleolytic cleavage of the concatemer, performed most likely by the terminase. This cut is either site specific (e.g., λ, ϕ105, T3, T7) or sequence independent in case of phages that package DNA by a headful mechanism (e.g., T4, P22, T1, P1, SPP1) (reviewed by Black, 1989). The terminase–DNA complex leftover unbinds the portal vertex and is thought to attach a new

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Table 1
SPP1 mutants used in this work

SPP1 mutant	Gene affected	Structures accumulated in SPP1 infections	Function of the protein	References
sus9	*9*	DNA-filled capsids	tail assembly	Becker et al., 1997
sus70	*1*	procapsids, tails	terminase small subunit	Chai et al., 1992
sus115	*6*	procapsids lacking portal protein, tails	portal protein	Tavares et al., 1992, Dröge et al., 2000
sus70sus115	*1* and *6*	procapsids lacking portal protein, tails	terminase small subunit portal protein	this work
sus128	*15*	empty expanded capsids, tails	head completion protein	Becker et al., 1997, Lurz et al., 2001

procapsid to perform the following cycle of encapsidation (Camacho et al., 2003; Gual et al., 2000). The portal pore is normally closed by the head completion proteins that prevent leakage of packaged DNA (Lurz et al., 2001; Orlova et al., 2003 and references therein).

The portal protein is a key component during viral chromosome packaging. Portal proteins are cyclical homo-oligomers with a central channel through which DNA movements occur (reviewed by Valpuesta and Carrascosa, 1994). They were proposed to be the mechanical device that pumps DNA to the interior of the viral capsid in a reaction fueled by the terminase ATPase activity during DNA packaging (Dube et al., 1993; Guasch et al., 2002; Moore and Prevelige, 2002; Simpson et al., 2000). This machinery is one of the strongest molecular motors described to date (Smith et al., 2001). A detailed description of the individual steps in which the portal protein participates is essential for a full understanding of the DNA packaging process.

Random mutagenesis of the portal protein (gp6) from the virulent *Bacillus subtilis* bacteriophage SPP1 combined with a screening strategy enabled the identification of mutants that arrest capsid morphogenesis at specific steps (Isidro et al., 2004). Here we report studies on selected mutants that block maturation of procapsids to DNA-filled capsids. The mutations can impair DNA encapsidation, capsid expansion, the headful gauge system, or the binding of head completion proteins. The portal protein is shown to play essential roles at most of the reactions occurring during encapsidation of the viral genome.

Results

Procapsid structures accumulate during SPP1 infection

A large excess of portal protein is produced in infected cells when compared to the amount required for assembly of the phage burst resulting from a SPP1 infection cycle (Tavares et al., 1995). Two possible scenarios can explain this observation: (i) the excess of portal is free in the cytoplasm implying a limiting step at the level of gp6 incorporation in the procapsid or procapsid assembly; (ii) or the portal protein is fully incorporated in procapsids and these structures or other intermediates of the morphogenetic pathway accumulate.

B. subtilis YB886 was infected with wild-type SPP1 to investigate whether any capsid assembly intermediate accumulated to significant amounts in the infected cell during a normal infection. We also monitored structures assembled during SPP1*sus*128 infection. SPP1*sus*128 is a nonsense mutant in gene *15* that codes for gp15, one of the proteins that binds to the portal vertex after DNA packaging to seal

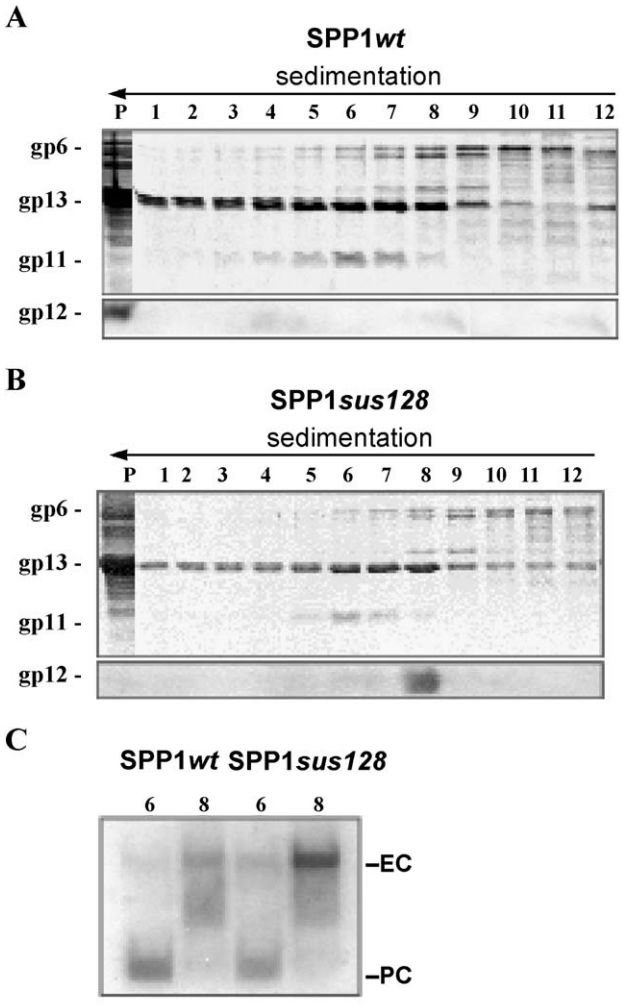

Fig. 1. Capsid-related structures produced in cells infected with SPP1*wt* and SPP1*sus128*. The structures were separated in 10–30% glycerol gradients (A—SPP1*wt*; B—SPP1*sus128*). Gradient fractions, numbered on top of the gel (P—pellet), were analyzed in SDS-PAGE and the protein content was visualized by Coomassie Blue staining. Gp12 was detected by Western blot (bottom panels in A and B). The position of gp6, gp13, gp11, and gp12 is labeled on the left side of A and B. Panel C shows the migration in agarose gels of procapsid-related structures that sediment in fractions 6 and 8 of SPP1*wt* and SPP1*sus128* gradients. EC–empty capsid; PC–procapsid.

the portal pore preventing DNA exit (Table 1; Orlova et al., 2003). Procapsids assembled in absence of gp15 undergo expansion and package DNA but the mature chromosome exits the capsid after the headful cleavage that terminates SPP1 DNA encapsidation (Becker et al., 1997; Orlova et al., 2003). These empty expanded capsids thus represent an abortive product from a late step of capsid morphogenesis. The structures produced in infections with SPP1*wt* and SPP1*sus*128 were resolved in 10–30% glycerol gradients under conditions that procapsid structures sediment to the center of the gradient while phage structures containing DNA are pelleted (Dröge et al., 2000; our unpublished results). Survey of gradient fractions in SDS-PAGE stained with Coomassie Blue showed a significant accumulation of particles that sediment in the center of the gradient (Figs. 1A, B). Most of these structures are found in fractions 6 and 7 that correspond to a peak of major capsid protein gp13, scaffolding protein gp11, portal protein gp6, and gp7 (Figs. 1A, B; data not shown). The presence of scaffolding protein reveals that the morphogenetic pathway was arrested before DNA translocation to the procapsid interior. Gp11 is the best marker for the sedimentation of SPP1 procapsids because the major capsid protein gp13 is found reproducibly more spread in the gradient (Dröge and Tavares, 2000). This feature of the SPP1 system is likely due to the presence of some polymorphic gp13 structures (electron microscopy observations not shown). Accumulation of procapsid particles in SPP1*wt* and SPP1*sus*128 infections shows that a significant proportion of these structures does not enter the following morphogenetic step that is DNA packaging. We are not yet in position to determine accurately their ratio relative to the product of the assembly pathway (phages or empty capsids in SPP1 wild-type or *sus*128 infections, respectively) because the gradient fractions and pellet are likely contaminated with some gp13 polymorphic structures.

Gp12 is a decoration protein that binds to SPP1-expanded capsids (A.C. Stiege, unpublished results). Results pre-

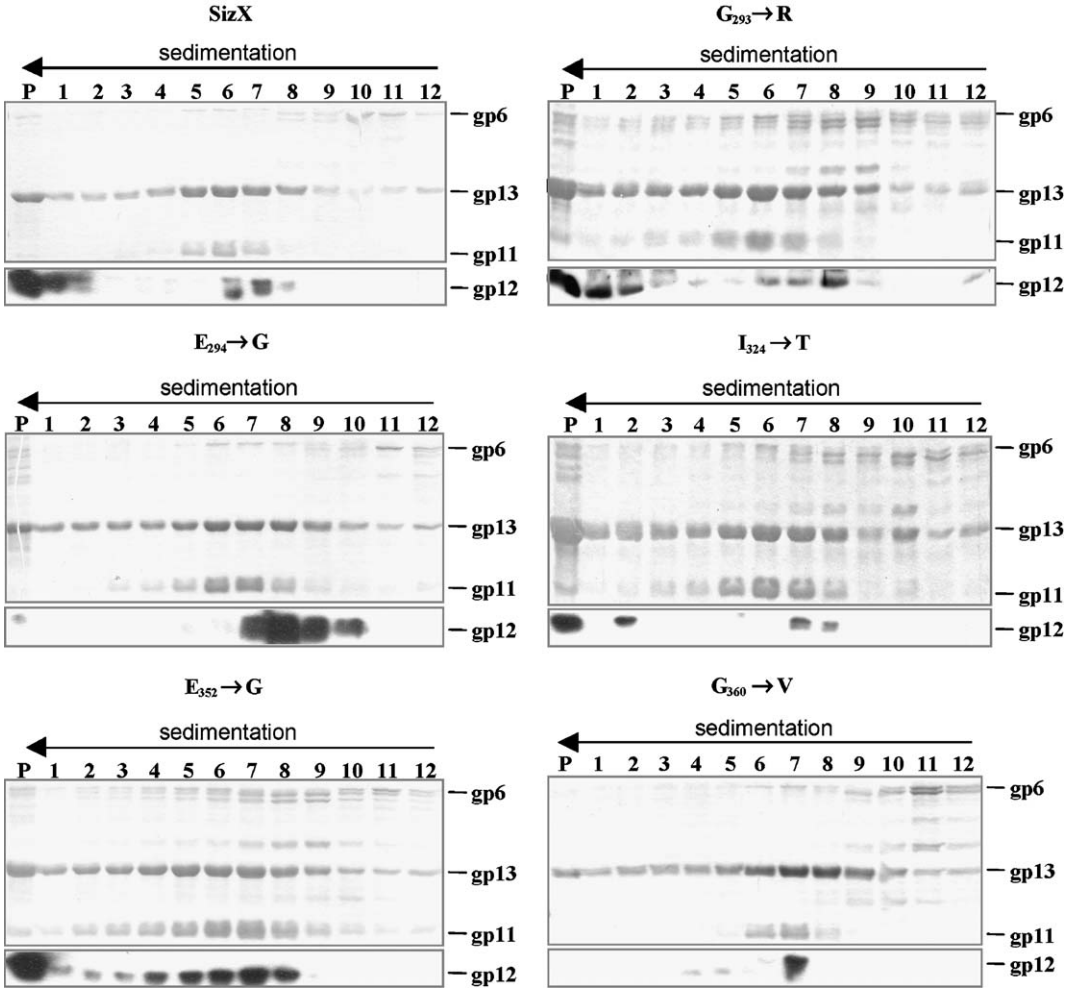

Fig. 2. Glycerol gradients of capsid-related structures carrying different gp6 mutant proteins. The amino acid substitution in gp6 is identified on top of each panel (SizX is the control run of structures carrying $gp6_{SizX}$). The gene *6* alleles carry the desired mutation and mutation *sizX* (see text). The gradient fractions, numbered on top of the gel (P—pellet), were analyzed in SDS-PAGE. The composition of the structures was determined by staining the SDS-PAGE gels with Coomassie Blue and by Western blotting developed with antibodies raised against gp12. The position of gp6, gp11, gp13, and gp12 proteins is labeled on the right side of the panels.

sented below confirm this correlation. Western blot analysis of the glycerol gradient fractions using antibodies against gp12 was done to investigate the distribution of structures that bound gp12 and have thus undergone capsid expansion that occurs during DNA packaging. In the wild-type infection, gp12 signal was only detected in the pellet of the gradient where sediment phage particles are the end product of the assembly pathway. In contrast, the structures assembled in SPP1*sus128* infections that contain gp12 sediment slightly slower than procapsids exhibiting a peak in fraction 8 of the gradient (Fig. 1B). They are most likely empty expanded capsids that lost their DNA after packaging was terminated because gp15 is not present for closure of the portal pore (Orlova et al., 2003). The lack of gp12 signal in the pellet indicates that DNA-filled structures are not assembled in SPP1*sus128* infections because of the block in stabilization of packaged DNA. Gp13 found in the pellet (Fig. 1B) is probably due to some large polymers of major capsid protein. The separation by agarose gel electrophoresis of structures present in fractions 6 and 8 of the glycerol gradients (Fig. 1C) confirmed that empty expanded capsids (EC in Fig. 1C) accumulate in SPP1*sus128* infections while procapsids (PC in Fig. 1C) are detected in wild-type and mutant infections. The absence of ECs in fraction 8 of the SPP1 wild-type gradient reveals that the stabilization of packaged DNA is an efficient step of capsid morphogenesis. Isopycnic centrifugation of phage-related structures assembled during SPP1 wild-type infections did not show presence of tailless phages (data not shown). DNA-filled capsids thus bind readily to tails in a normal infection to yield full virions. The ensemble of the results shows that DNA packaging is the rate-limiting step in SPP1 morphogenesis.

Screening of mutations in the SPP1 portal protein that impair different reactions during DNA packaging

Following that DNA packaging is the limiting step in SPP1 morphogenesis, we used our library of mutations in the SPP1 portal protein gp6 to characterize different stages of this process. Gp6 mutant proteins carrying each of the 39 single amino acid substitutions that arrest DNA packaging but do not affect procapsid assembly in an in vivo complementation assay (Isidro et al., 2004) were screened for the reaction they affect during encapsidation. The viral structures assembled in presence of the gp6 mutant forms were resolved in glycerol gradients. Their composition was determined by Coomassie Blue staining and Western blot although the type of structures present was checked by agarose gel electrophoresis (data not shown). Generation of DNA molecules with unit-chromosome length that result from complete cycles of DNA packaging was detected by pulse-field gel electrophoresis (Isidro et al., 2004) and their stable packaging was assayed in DNase protection assays (data not shown). After the screening, we selected five gp6 missense mutants for further studies (G_{293}→R, E_{294}→G, I_{324}→T, E_{352}→G, and G_{360}→V) that are representative of the various phenotypes found and are thus likely candidates to arrest different reactions during DNA packaging. All these gp6 forms were incorporated in the procapsid structure at normal levels (Isidro et al., 2004).

Capsid-related structures that accumulate during infection have different properties depending on the mutant portal protein present in the structure

B. subtilis cells expressing the mutant gene *6* alleles under analysis linked to the *sizX* mutation (gp6 E_{424}→K; Tavares et al., 1992) were infected with SPP1*sus115*, which is deficient in gp6 production (Isidro et al., 2004). Under these conditions, the gp6 version present in the cell determines the phenotype associated to portal protein function (Isidro et al., 2004; Tavares et al., 1995). The capsid-related structures were purified in glycerol gradients and their protein composition was determined (Fig. 2). Most of the procapsid-related structures sedimented in the middle of the

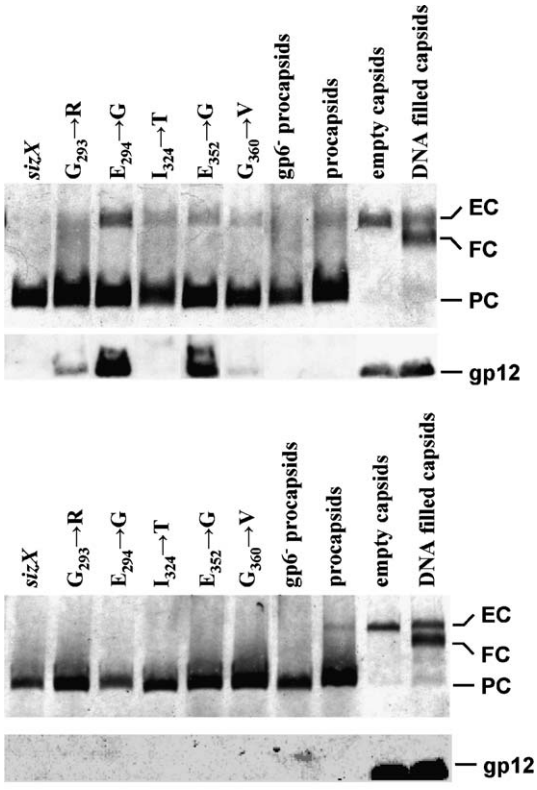

Fig. 3. Agarose gels of capsid-related structures assembled in *B. subtilis* strains coding for gp6 mutant proteins that were infected with SPP1*sus115* (A) or with SPP1*sus70sus115* (B). Gp6$^-$ procapsids, procapsids, empty capsids, and DNA-filled capsids were purified from extracts of *B. subtilis* strains infected with SPP1*sus115*, SPP1*sus70*, SPP1*sus128*, and SPP1*sus9*, respectively (Table 1). The fraction of the glycerol gradient enriched in capsid material was separated on 1.75% agarose gels. Western blots of the same fractions developed with antibodies raised against gp12 are shown below the agarose gels. The position of procapsids (PC), empty capsids (EC), DNA-filled capsids (FC), as well as the gp12 position, is labeled on the right side of the gels.

gradient (fractions 6 and 7; Fig. 2). In case of the mutant E_{352}→G, the distribution of SPP1 proteins along the gradient was reproducibly more dispersed than that observed in all other gradients as can be best appraised for gp11 and gp12, marking the position of procapsids and empty expanded capsids sedimentation, respectively (Fig. 2).

A strong signal in anti-gp12 Western blots was found for structures carrying mutant gp6 E_{294}→G and E_{352}→G. In case of gp6 E_{294}→G, the distribution of proteins in the gradient was similar to the one found for structures produced during SPP1*sus128* infections with a clear peak of gp12 in fraction 8 (Figs. 1 and 2). In contrast, gp12 was found spread in the gradient of assembly intermediates containing gp6 E_{352}→G confirming the heterogeneity of the structures assembled in presence of this mutant portal protein. The faint gp12 signal observed for structures with gp6 G_{293}→R, I_{324}→T, G_{360}→V, and with the control SizX corresponds probably to background caused by empty capsids of infective phages (Fig. 2). Detection of significant amounts of gp12 in the pellet of gradients showed that capsids filled with DNA and phages are assembled in presence of gp6 G_{293}→R, I_{324}→T, E_{352}→G, and of the control SizX (Fig. 2).

In order to investigate if the structures in the center of the gradient that bound gp12 were expanded, the richest fractions of capsid-related structures were analyzed in agarose gels. The procapsid band is always the most abundant species (Fig. 3A) indicating that a significant proportion of procapsids are not used for the DNA packaging reaction. The finding of empty expanded capsids in the population of structures carrying gp6 E_{294}→G correlates well with the presence of gp12 (Figs. 2 and 3A). A weak band of expanded capsids (Fig. 3A) and a spread signal of gp12 were detected reproducibly in the gradient of structures containing gp6 E_{352}→G (Figs. 2 and 3A). The presence of a faint band of material comigrating with empty capsids that is in found in some of the other gradients (Fig. 3A) depends on the preparation of structures used. Its origin remains to be established.

Presence of expanded capsids that have gp12 bound may either be related to expansion or to an anomalous procapsid assembly process leading to exposure of gp12 binding sites in the gp13 lattice. To distinguish between these two possibilities, the strains bearing a mutated copy of gene *6* under analysis were infected with the double mutant SPP1*sus115sus70*. This mutant is deficient in the production of both the portal protein and the terminase small subunit (Chai et al., 1992). In SPP1*sus115* infections under complementation conditions, the progeny has the phenotype conferred by the gp6 form produced in the host cell. In SPP1*sus115sus70* infections of the same host, the progeny obtained only shows a phenotype bestowed by the gp6 mutant form when the mutation affects a step before interaction of the terminase with the portal vertex. If a latter step is blocked, morphogenesis is arrested at the step of terminase binding to DNA that precedes initiation of DNA encapsidation (Chai et al., 1992, 1995). Structures obtained in infections with SPP1*sus115sus70* did not run at the level of empty expanded capsids in agarose gels and no gp12 was detected (Fig. 3B). Capsid expansion and gp12 binding to the capsid lattice thus occur in all cases after DNA packaging initiation confirming that the effect of gp6 mutations in these processes reveals a defect in DNA encapsidation.

Fate of SPP1 DNA during infection

DNA packaging into procapsids containing gp6 mutant forms was tested in vivo using the complementation assay described above. Total DNA and DNase-treated samples were processed 22 min after SPP1 infection and resolved by

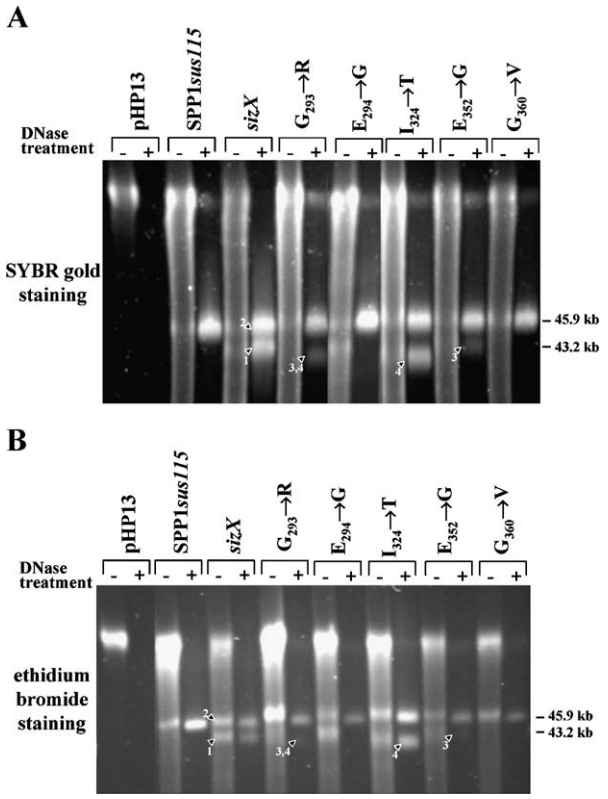

Fig. 4. Generation of unit-length viral chromosomes in *B. subtilis* strains infected with SPP1. Total DNA from noninfected or from infected cells was resolved by PFGE in 1% agarose gels stained with SYBR Gold (A) or with ethidium bromide (B). Samples untreated (−) and treated with DNase (+) were analyzed. Host cell DNA is visualized in the samples from noninfected cells bearing the vector pHP13. The other samples originate from cells infected with SPP1*sus115*. SPP1*sus115* is an infection of the nonpermissive YB886 strain. The other gel lanes are labeled according to the mutation in gp6 codified by the gene *6* allele expressed in the infected YB886 cell. The gene *6* alleles carry the desired mutation and mutation *sizX* (see text). Mature DNA purified from wild-type (DNA SPP1*wt*) and SPP1*sizX* (DNA SPP1*sizX*) virions was used as size standard in the PFGE (sizes indicated on the right). Arrowheads show the mature DNA produced upon complementation with the *sizX* allele (1), mature DNA resulting from background effects under our experimental conditions (see text) (2), DNA packaged in presence of gp6 mutant forms that caused a low efficiency phenotype (3), and DNA packaged in presence of a $gp6_{Siz}$ mutant protein (4).

Table 2
Phenotypes associated to the gp6 mutations characterized in this work

Amino acid substitution	Titer (% from control)	Procapsid assembly	Purified capsid-related structures		Agarose gels		DNA packaging			Phenotype
			gp12 binding (fraction of PC* structures)	gp12* binding (fraction of EC* structures)	PC	EC	Mature DNA	DNase protection	Mature DNA size	
SizX (control)	100%	normal	–	–	++++	–	++++	++++	normal	
G_{293}→R	1.6%	normal	–	+	++++	–	+	++	<	low packaging efficiency small chromosome
E_{294}→G	0.7%	normal	–	+++++	++++	++++	++++	–	normal	no packaged DNA protected
I_{324}→T	16%	normal	–	+	++++	+	++++	++++	<<<	small chromosome
E_{352}→G	2.7%	normal	+++	+++	++++	++	+	++	normal	low packaging efficiency
G_{360}→V	2.5%	normal	+	+	++++	+	–	–	–	no packaging

The gp6 single amino acid substitutions caused by missense mutations in gene *6* are shown on the left column. The severity of the mutations was estimated in complementation assays by titration of SPP1*sus115* phages in strains bearing plasmids with the gene *6* mutant alleles and expressed as percentage of the titer obtained with the strain coding for SizX (second column). Production of procapsids with normal composition is based on Isidro et al. (2004). The phenotype of each mutation was determined based on the protein content of capsid-related structures (Figs. 2 and 3), the migration of these structures in agarose gels (Fig. 3), and characterization of viral DNA present in infected cells (Fig. 4). Relative signal intensity vary from "–" (no signal) to "++++" (maximum signal). *PC, fraction 6 of glycerol gradients; EC, fraction 8 of glycerol gradients (Fig. 2). The relative size of the DNA encapsidated was expressed by comparison with the *sizX* chromosome size, from "<<<" (clearly smaller) to "normal" (*sizX* chromosome size).

pulse-field gel electrophoresis (PFGE). SPP1 infection leads to a significant increase in the amount of cellular DNA as shown by comparison of total DNA samples from *B. subtilis* noninfected (pHP13 in Fig. 4) and infected cells (other samples in Fig. 4). Unit-length SPP1 chromosomes that are detected as discrete bands in the lanes of infected cells samples result from complete DNA packaging cycles. Their protection from DNAse indicates that the viral chromosome is stably packaged inside the viral capsid. Gel staining with SYBR Green Gold was used to detect low amounts of stably packaged DNA but was not appropriate to observe sharp bands in the total DNA samples due to the high amount of DNA present (Fig. 4A). Bands in these samples were visualized with the less sensitive ethidium bromide staining.

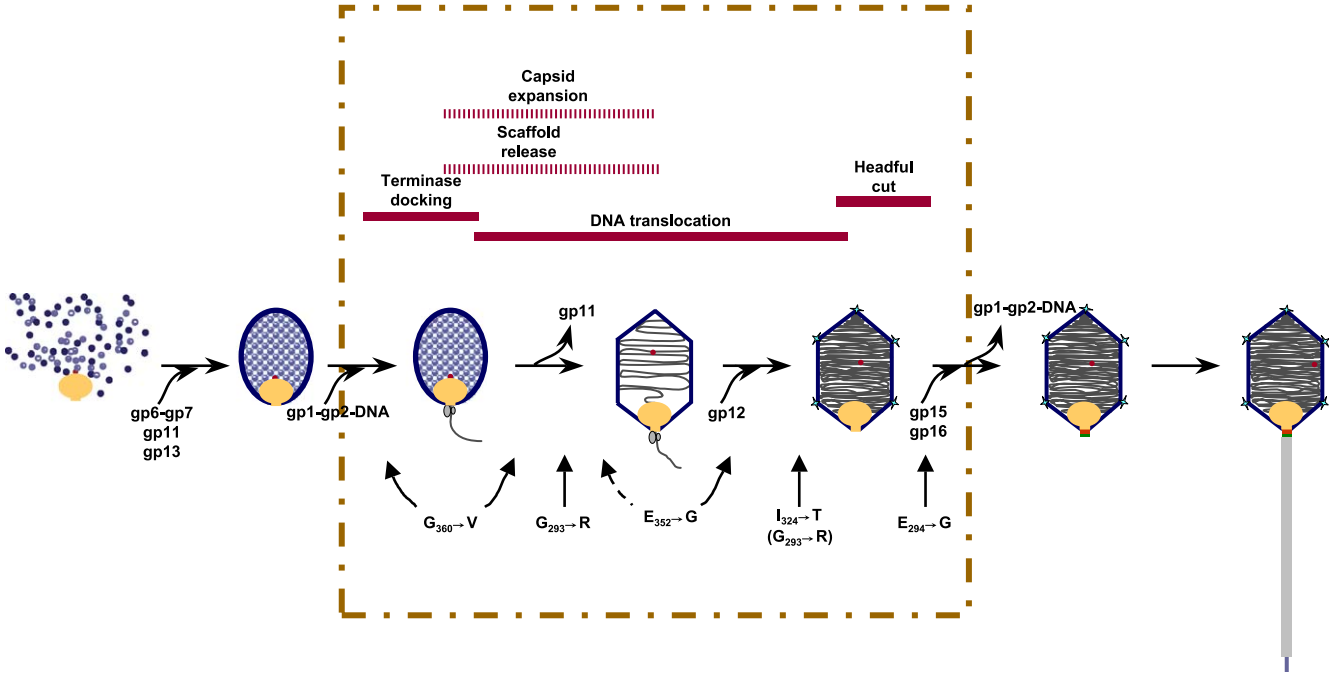

Fig. 5. Schematic representation of the SPP1 morphogenetic pathway. The steps of DNA packaging in which gp6 was shown to participate are detailed inside the dashed box. The step affected by each mutation characterized in this work is identified below the pathway scheme. The solid red bars on top of the pathway represent the span of each reaction during DNA packaging although the striped bars indicate the tentative timing of other steps. Gp6 is presented in yellow, gp7 in red, gp11 as open blue circles, gp12 as stars, gp13 as filled blue circles and as a thick blue line representing the capsid lattice, the head completion proteins as orange and green bars, the terminase as gray circles, and DNA as a gray line.

The upper band of unit-length SPP1 DNA observed in PFGE corresponds to background due to input phages or recombination events during infection (arrowhead 2 in Fig. 4; Isidro et al., 2004). In order to allow specific identification of DNA resulting from de novo packaging, the plasmid present in the infected cell expresses gene *6* carrying the mutation under analysis linked to the *sizX* allele. The *sizX* mutation causes an undersizing of the packaged DNA (Tavares et al., 1992). Therefore, the lower band of SPP1 chromosomes in the PFGE (arrowhead 1 in Fig. 4) corresponds to DNA molecules packaged de novo that are smaller than those from input phages. Presence of the *sizX* allele does not hamper identification of other *siz* mutations because combination of this type of mutations appears to have an additive effect in chromosome undersizing (Tavares et al., 1995; see below). The electrophoretic mobility in PFGE of SPP1 chromosomes packaged de novo reveals their size while the intensity of the corresponding band provides an estimate of the DNA packaging efficiency (number of complete DNA packaging cycles that occurred during infection). The comparison between DNase-treated and nontreated samples identifies cases in which the packaging cycle was successfully finished but the viral chromosome leaked from the capsid afterwards.

Characterization of the gp6 mutations allowed the distinction between four different packaging mutant phenotypes: (i) no mature unit-length viral chromosomes detected (G_{360}→V); (ii) reduced packaging efficiency (G_{293}→R, E_{352}→G, arrowheads 3 in Fig 4); (iii) undersized mature chromosomes (G_{293}→R, I_{324}→T, arrowheads 4 in Fig. 4); and (iv) mature DNA is present but not protected against DNase treatment (E_{294}→G) (Fig. 4).

The phenotypes associated to the gp6 mutations (Table 2) show that the portal protein plays a role in different reactions during the DNA packaging process (Fig. 5).

Discussion

DNA packaging is a limiting step in SPP1 morphogenesis

Accumulation of procapsids in SPP1 infections revealed a limiting step in the SPP1 assembly pathway. These structures have the normal procapsid composition characterized by the presence of the scaffolding protein that has to leave the structure to provide the space for accommodating the DNA during packaging (Fig. 5). Morphogenesis is therefore arrested before this event, most probably at the beginning of viral chromosome encapsidation. Presence of concatemeric SPP1 DNA in infected cells, accessible to DNase digestion (Fig. 4), shows that the amount of substrate for packaging is not a limiting factor. Several lines of evidence suggest that formation of initiation complexes for processive packaging of SPP1 DNA is the critical step. A limiting number of packaging initiation events was found to occur during SPP1 infection (Tavares et al., 1996). Assembly of initiation complexes requires terminase loading to the substrate DNA concatemer and docking of this complex to the procapsid portal vertex. Studies in vitro indicate that the SPP1 packaging machinery remains associated to the same concatemeric DNA molecule throughout phage assembly implying that the terminase does not normally switch between substrate DNA molecules (Dröge and Tavares, 2000). The terminase protein exhibits a labile behavior (Dröge and Tavares, 2000; Poteete et al., 1979) and its amount in the cell is tightly regulated (Chai et al., 1997), further indicating that assembly of initiation DNA packaging complexes is a well-controlled step that is likely rate-limiting in the DNA packaging reaction. We cannot distinguish now if this is because of (i) a deficient assembly of terminase–DNA complexes, (ii) because these complexes are present in limiting amounts in the infected cell, (iii) because the terminase–DNA complex interaction with the portal vertex that precedes DNA translocation to the procapsid interior occurs inefficiently, and (iv) because procapsids that accumulate in the infected cell are not competent for DNA packaging in spite of their normal composition.

The portal protein participates in different steps of the DNA packaging process

Packaging of the bacteriophage SPP1 chromosome engages a complex sequence of reactions occurring at the specialized portal vertex that is the door for DNA entry and exit of the viral capsid. The DNA packaging machinery of tailed bacteriophages, herpes viruses, and Tectiviridae assembles at this vertex and translocates the viral chromosome to the capsid interior using related molecular mechanisms (Black, 1989; Gowen et al., 2003; Newcomb et al., 2001). The portal protein cyclical oligomer is organized around a central channel through which DNA movements occur. Characterization of a set of mutations in the portal protein gp6 of bacteriophage SPP1 showed that the portal assembly participates in most of the reactions that lead to stable packaging of the viral genome (Table 2, Fig. 5). The step affected was identified by accumulation of assembly products that are normally not present in high amounts in the infected cell and defects on the production of stably packaged DNA. The sequential events in DNA packaging monitored in our assays were (i) capsid expansion and gp12 binding that occur after initiation of DNA packaging; (ii) termination of the DNA packaging cycle that generates unit-length chromosomes; (iii) the size of mature chromosomes determined by a headful sensor mechanism that triggers cleavage of the substrate DNA terminating the encapsidation cycle; (iv) stabilization of the packaged DNA that leads to its protection from DNase. The phenotype of the different mutations characterized here allowed to position the (partial) block and defects they cause at specific steps of the viral assembly pathway (Fig. 5).

The phenotype of a mutation that blocks an early step of DNA packaging is illustrated by gp6 G_{360}→V: procap-

sid structures accumulate in the cytoplasm although neither expanded capsids nor unit-length chromosome DNA are detected among the structures assembled (Figs. 2–4). The mutation arrests DNA packaging before capsid expansion is triggered. It affects most probably the interaction of gp6 with the terminase–DNA complex at the critical step of packaging initiation, the beginning of DNA translocation, or the release of the scaffolding proteins from the procapsid interior (Fig. 5). Mutation G_{293}→R exhibits a similar phenotype, but in this case, a small amount of undersized chromosomes is stably packaged (Fig. 4A; Table 2). This implies that the block that occurs before capsid expansion is overcome in some cases allowing encapsidation to proceed to the end yielding structures with stably packaged DNA. In these cases, the headful cleavage that terminates the DNA encapsidation cycle occurs prematurely (see below).

A population of structures with heterogeneous sedimentation behavior is assembled in the presence of gp6 E_{352}→G (Fig. 2). Structures containing the scaffolding protein gp11 (the marker for procapsids) and, more evidently, the capsids that bind gp12 show a spread sedimentation behavior (Fig. 2). A small amount of empty expanded capsids was detected in agarose gel electrophoresis (Fig. 3). Therefore, it is likely that a mix of capsid-related structures carrying gp6 E_{352}→G accumulate during infection. This feature is a consequence of DNA packaging because only normal procapsids accumulate when packaging initiation is blocked in infections with a mutant deficient in terminase production (SPP1*sus115sus70*; Fig. 3B). Some structures might still maintain the procapsid shape though packaging was initiated as found in other phage systems in which capsid expansion occurs after a certain amount of DNA has been packaged (Bjornsti et al., 1983; Hohn, 1983; Jardine and Coombs, 1998; Shibata et al., 1987). The presence of both the gp11 scaffolding protein and gp12 in the fast-sedimenting capsids suggests that these particles have never contained a headful of DNA. This phenotype, found exclusively with gp6 E_{352}→G structures, reveals that the mutant gp6 causes most likely a defect after initiation of DNA packaging and before completion of the DNA packaging cycle. The block in packaging is only partial because a few DNA encapsidation cycles were terminated as shown by the faint band of unit-length viral chromosomes detected in PFGE (Fig. 4). Gp6 E_{352}→G affects most likely the DNA translocation process. We hypothesize that the mutation E_{352}→G reduces significantly the rate of DNA translocation to the capsid interior either by directly affecting the DNA pumping motor or by blocking motion of DNA through the gp6 central channel. The structures sedimenting to different positions in the gradient would correspond to capsids filled with variable amounts of DNA that represent intermediates trapped during DNA translocation. A fraction of these intermediates expanded and bound gp12. The variability in size of the packaged DNA fragments could account for our inability to detect their presence both in PFGE (Fig. 4) and in conventional agarose gels (data not shown) in spite of significant effort using different detection methods. We are presently developing an in vitro packaging system with purified components to further investigate the hypothesis proposed. Procapsids carrying gp6 E_{352}→G are a highly promising tool to study the DNA translocation machine working in "slow-motion" using such system.

Mutations E_{352}→G, G_{293}→R, and several other single amino acid substitutions in the SPP1 portal protein (Isidro et al., 2004) exhibit a low packaging efficiency phenotype characterized by occurrence of a reduced number of complete packaging cycles during infection. This phenotype is due to an inefficient reaction at any of the steps of the DNA packaging cycle. Therefore, the various mutations identified are likely affecting distinct reactions in the packaging process as exemplified by the unique features associated to gp6 E_{352}→G. Mutations causing a low efficiency of DNA packaging were also found in the terminase of phages T3 and λ (Kimura and Fujisawa, 1991; Morita et al., 1995; Yeo and Feiss, 1995; and references therein), revealing that the portal and terminase proteins are both central components of the viral DNA packaging motor.

DNA packaging in SPP1 is terminated by the headful cleavage that occurs after a threshold amount of DNA is reached inside the capsid. The portal protein was previously shown to be a central component of the headful sensor in bacteriophages P22 (Casjens et al., 1992) and SPP1 (Tavares et al., 1992). The amino acid substitution I_{324}→T causes a significant undersizing of the mature SPP1 chromosomes (*siz* phenotype) although packaging efficiency is normal (Fig. 4). Phage particles are assembled under these conditions but only ~16% are infectious (Table 2) although the others are defective because they do not carry a complete viral genome (Tavares et al., 1992). The mutation affects exclusively the headful sensor mechanism. Other amino acid substitutions in gp6 cause both a reduction in packaging efficiency and undersizing of the packaged DNA (e.g., G_{293}→R, Fig. 4), the mutation in the same residue I_{324}→V and others (data not shown; Isidro et al., 2004), which could imply a coupling between the headful sensor and the DNA translocation mechanisms. However, the finding of mutations that caused exclusively one of the phenotypes (low efficiency of packaging: E_{352}→G; smaller chromosome phenotype: I_{324}→T; Fig. 4; see also Isidro et al., 2004) rather suggest that headful cleavage is controlled by a specific mechanism independent of the DNA translocation motor performance (Isidro et al., 2004).

Stabilization of the DNA-filled capsid is the last step of capsid assembly. In most bacteriophages studied, the addition of proteins to the portal vertex closes the portal pore, preventing chromosome leakage and providing an interface for tail attachment or assembly (T4, Coombs and Eiserling, 1977; P22, Strauss and King, 1984; λ, Padmanabhan et al.,

1972; SPP1, Lurz et al., 2001; Orlova et al., 2003). The mutation E_{294}→G does not affect generation of SPP1 mature chromosomes but those are sensitive to DNase treatment (Fig. 4). Capsids containing this mutant gp6 form expand and bind gp12 (Figs. 2 and 3, Table 2). The failure to detect gp12 signal in the gradient pellet shows that no complete phages were formed. Thus, this mutant portal oligomer carries out its functions during DNA packaging but the encapsidated chromosomes are not stably maintained inside the capsid. The phenotype observed is similar to the one found for infection with SPP1*sus128* that does not produce the head completion protein gp15 (Fig. 1, Orlova et al., 2003). Because gp15 forms a ring underneath gp6 in the connector structure (Lurz et al., 2001; Orlova et al., 2003), the E_{294}→G substitution hampers most likely the interaction between the two proteins. This is the only mutation in our library that blocks specifically this late step of capsid assembly. It is interesting to note that the residue mutated is adjacent to G_{293} whose substitution by arginine impairs an early step of DNA packaging (Fig. 5) demonstrating that the same region of gp6 plays a role in different reactions during DNA packaging.

Our study identifies reactions in the sequential steps of the viral DNA packaging cycle that can be disrupted by mutations in the portal protein. This component of the DNA packaging machinery is shown to be essential in most of those steps. The remarkable similarities found in the DNA packaging mechanisms of prokaryotic and eukaryotic viruses that use portals for entry and exit of the viral genome from their capsids (Gowen et al., 2003; Hendrix, 1999; Newcomb et al., 2001) suggest strongly that their portal proteins play similar functions to the ones found for gp6 of bacteriophage SPP1.

Material and methods

Bacterial strains, bacteriophages and plasmids

SPP1*sus70*, SPP1*sus115*, SPP1*sus128*, and SPP1*sus9* were previously described (Table 1; Becker et al., 1997; Chai et al., 1992; Tavares et al., 1992). SPP1*sus115sus70* was constructed by a standard phage cross procedure and screening with complementing plasmids. The plasmid vector used in all genetic manipulations is a derivative of plasmid pHP13 (Haima et al., 1987) carrying the complete gene *6* cloned as a *Hpa*I–*Bcl*I fragment of SPP1 DNA (Tavares et al., 1992) (coordinates 2267–3917 in the SPP1 sequence, GenBank accession number, X97918; Alonso et al., 1997). Gene *6* was cloned downstream of the inducible promoter $P_{N25/0}$ (Le Grice, 1990; Tavares et al., unpublished results). Expression was controlled by the LacI repressor coded by plasmid pGB3 in *Escherichia coli* (strain ECE89 of the Bacillus Genetic Stock Center) or pEB104 in *B. subtilis* (Leonhardt and Alonso, 1998). Missense mutations in gene *6* were transferred from pFiFΔAcc (Isidro et al., 2004) to the vector by subcloning of fragments *Pfl*MI–*Asp*718 (coordinates 3040–3244 of the SPP1 sequence) (G_{293}→R, E_{294}→G) or *Asp*718–*Pst*I (coordinates 3244–3917 of the SPP1 sequence; the *Pst*I site is from the vector polylinker) (I_{324}→T, E_{352}→G, G_{360}→V). The replacement strategy is schematized in Fig 1 of Isidro et al. (2004). Cloning in this vector allowed 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 (Isidro et al., 2004).

Distribution of procapsid-related structures in glycerol gradients and determination of their protein content

B. subtilis clones bearing plasmids encoding the gp6 mutant forms under analysis were infected with SPP1*sus115* phages to an input multiplicity of 5. The capsid-related structures present in the infected cells were resolved in 10–30% glycerol gradients and their protein content was analyzed as described (Becker et al., 1997; Dröge et al., 2000). Rabbit antibodies against gp12 and gp7 kindly provided by A.C. Stiege and J.C. Alonso, respectively, were used for the detection of those proteins in Western blots.

Visualization of intermediates of SPP1 capsid morphogenesis in agarose gels

Serwer and Pichler (1978) had shown that the migration of phage particles in an agarose matrix in the presence of an electric field was not dependent on the internal content of the capsid (core or DNA) but rather on the particle size and net surface charge. This methodology was used to examine capsid-related structures produced by SPP1*sus* mutants arrested at specific steps of capsid morphogenesis and those containing the mutant gp6 forms under analysis. The procedure and running conditions were as described (Dröge et al., 2000).

Analysis of DNA packaging

B. subtilis cells bearing the gene 6 alleles under analysis were infected with SPP1*sus115* to an input multiplicity of 10. DNA present in the infected cells at 22 min post-infection was analyzed by PFGE as described (Isidro et al., 2004), with the exception that half of the agarose blocks were treated with a lysis buffer lacking DNase (6 mM Tris–HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 1 M NaCl; 30 mM *N*-Lauroyl-sarkosine; 1 μg/ml RNase; 10 μg/ml lysozyme). PFGE gels were stained with SYBR Gold (Molecular Probes, Leiden, The Netherlands) or with ethidium bromide.

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