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The Functions of the N Terminus of the ϕX174 Internal Scaffolding Protein, a Protein Encoded in an Overlapping Reading Frame in a Two Scaffolding Protein System

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ϕX174 utilizes two scaffolding proteins during morphogenesis, an internal protein (B) and an external protein (D). The B protein induces a conformational change in coat protein pentamers, enabling them to interact with both spike and external scaffolding proteins. While functions of the carboxyl terminus of protein B have been defined, the functions of the amino terminus remain obscure. To investigate the morphogenetic functions of the amino terminus, several 5′ deleted genes were constructed and the proteins expressed *in vivo*. The ΔNH_2 B proteins were assayed for the ability to complement an ochre B mutant and defects in the morphogenetic pathway were characterized. The results of the biochemical, genetic and second-site genetic analyses indicate that the amino terminus induces conformational changes in the viral coat protein and facilitates minor spike protein incorporation. Defects in conformational switching can be suppressed by substitutions in the external scaffolding protein, suggesting some redundancy of function between the two proteins.

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Keywords: ϕX174; scaffolding protein; morphogenesis; procapsid

Introduction

Bacteriophage ϕX174 employs two scaffolding proteins during morphogenesis, an internal and the external species. Together these two proteins perform the functions associated with the larger scaffolding proteins found in one scaffolding protein systems.[1] They ensure the morphogenetic integrity, nucleate capsid assembly[2] and inhibit off-pathway aggregation reactions.[3] The ϕX174 morphogenetic pathway has been extensively characterized (Figure 1). The first detectable intermediates are the 9 S and 6 S particles, respective pentamers of coat and spike proteins.[4] Five internal scaffolding proteins, protein B, bind to the underside of coat pentamers, inducing a conformational change that enables the pentamers to interact with the major spike and external scaffolding proteins.[3–5] The external scaffolding protein, protein D, assembles 12 of these pentameric intermediates into the procapsid.[6] The single-stranded DNA replication and packaging complex then docks at a 2-fold axis and fills the particles.[7] DNA packaging is associated with the gain of the DNA binding protein J and the extrusion of internal scaffolding protein to form the infectious provirion.[8] Maturation completes with the closing of the 3-fold related pores, a radial collapse of coat pentamers, and the dissociation of the external scaffolding protein, yielding the mature 114 S mature virion.[9] If particles assemble without a full complement of the minor spike protein, improperly filled 110 S particles are formed.[10] Loss of the external scaffolding protein then results in the formation of uninfectious 70 S particles.

The internal scaffolding protein of ϕX174 contains 120 amino acid residues and presumably assumes numerous orientations during morphogenesis as evidenced by genetic and structural data.[5,11–13] Internal scaffolding proteins from ϕX174, α3, and G4 can cross-function despite only 30% similarity.[12] Similarity is limited almost

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Abbreviation used: eop, efficiency of plating.

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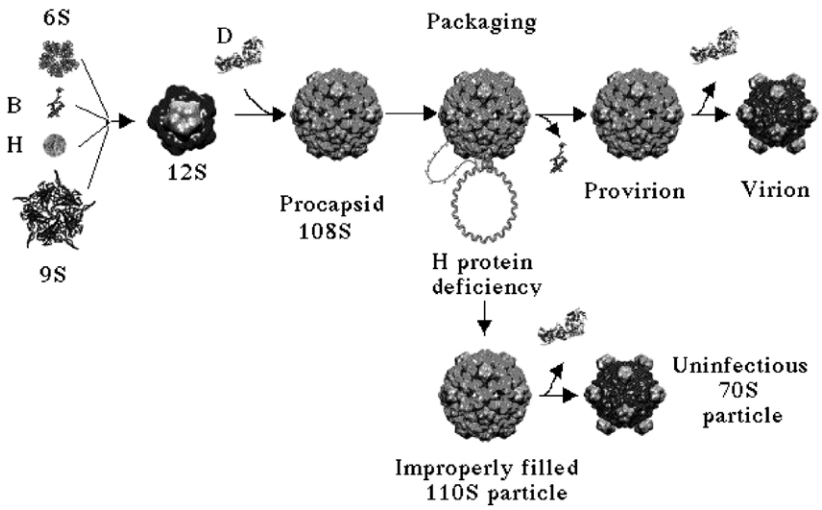

Figure 1. The ϕX174 morphogenetic pathway, including the off pathway production of defective 110 S and 70 S particles.

exclusively to the C-terminal halves of the proteins. This region is well ordered in the X-ray structure and determines coat protein specificity and recognition.[5,11,13] B protein density in the crystal model is relatively unordered for the first 58 amino acid residues with the exception of ten most N-terminal amino acid residues, which may make B–B contacts across 2-fold axes of symmetry. In a 15 Å resolution cryo-image reconstruction, the C^{α} backbone can be traced from the C terminus to a large α-helix starting at residue 67 and ending at residue 58, at which point the protein appears to veer toward the 5-fold axis of symmetry (Figure 2).

The lack of ordered density in the N-terminal half of the protein and the inability to isolate B protein missense mutations with defective phenotypes has complicated both genetic and structural analyses. In an effort to elucidate the possible functions of the N terminus, a series of N-terminal deletion genes were constructed and the proteins expressed *in vivo*. The results of this analysis suggest that the N terminus facilitates the incorporation of the minor spike protein and participates in the conformational switching of coat protein pentamers. The results of a second site genetic analysis indicate that this latter function is performed in concert with the external scaffolding protein.

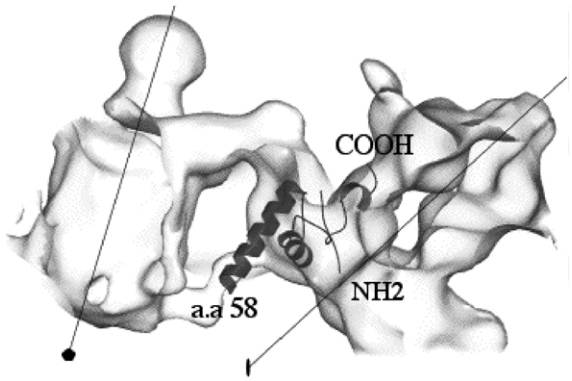

Figure 2. The structure of the internal scaffolding protein. The atomic structure of the α3 B protein modeled into a 15.0 Å image reconstruction. The large α-helix spans amino acid residues 58-Δ67. Residues before 58 veer toward the 5-fold axis of symmetry, represented by the pentagon. The 2-fold axis is represented with an oval.

Results

Characteristics of ΔNH_2-terminal internal scaffolding proteins

Previously two ΔNH_2-terminal internal scaffolding proteins were assayed for their ability to support ϕX174 morphogenesis.[13] One protein lacked the first ten amino acid residues that may participate in B–B interactions across 2-fold axes of symmetry while the other consisted only of the highly ordered C-terminal residues.[5,11] While the deletion of the first ten amino acid residues appeared to have little effect on the production of infectious particles, the larger deletion was unable to support morphogenesis. To determine the minimal COOH-terminal length of B protein required for complete morphogenesis, additional 5′ deleted genes, Δ22, Δ27, Δ32, Δ38, Δ43, Δ48, Δ53, and Δ58 (Figure 3), were cloned behind an inducible promoter and assayed for the ability to complement a B^{-} ochre mutant over the entire range of ϕX174 growth (Table 1). In these experiments, sup° cells expressing the cloned ΔB genes were infected by the *och*(*B*)*Q3* mutant. Proteins lacking up to 27 amino acid residues were capable of supporting morphogenesis in these plaque assays. While the Δ32 and Δ38 B proteins did not complement, proteins with more extensive deletions (Δ43 and Δ53) were functional.

The ΔB proteins that displayed no complementation activity were analyzed for inhibitory action, or dominant lethal phenotypes, in the presence of the wild-type protein. Wild-type ϕX174 was plated on cells expressing these proteins (data not shown). Expression had no effect on the ability of the

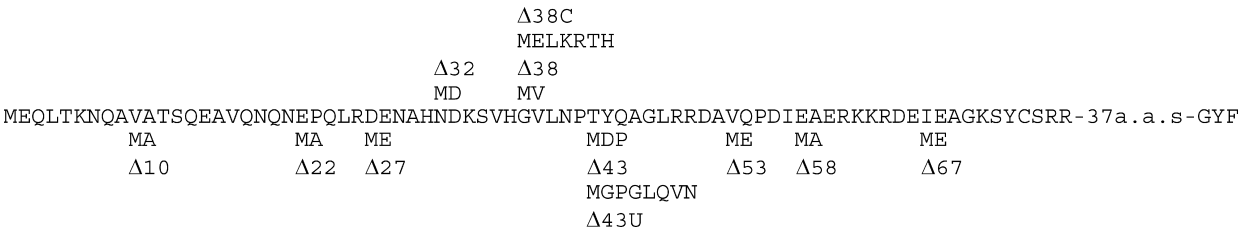

Figure 3. The amino acid sequence of the ϕX174 internal scaffolding protein. The first two residues of the Δ proteins are depicted above and below the line. In some cases two residues had to be mutated to place an *Nco*I site, which contains an ATG start codon, into the gene. Additional residues for the Δ38C and Δ43U proteins (see the text for description) are also given.

wild-type phage to form plaques or on plaque morphology. This assay was repeated using an *am(B)* mutant in a *supF* tRNA suppressor cell line (BAF8) that also contained a plasmid expressing either Δ38 or Δ67 protein. The *supF* tRNA molecule operates at a 28% efficiency;[14] therefore, there should be a lower concentration of full-length B protein produced in *am*(*B*) infected cells as compared to wild-type infected cells. Again no inhibition was observed (data not shown) in either plating efficiency or plaque morphology. The results of these studies suggest that the Δ38, Δ67 proteins either do not interact with the viral coat protein, cannot effectively compete with it, or a full complement of wild-type B protein is not required for morphogenesis, at least within the detection limits of these qualitative plating assays.

In an effort to explain the "zone of inactivity" found between amino acid residues 32 and 38, the NH_2 terminus of each ΔB protein was examined (Figure 3). The N-terminal ends of the Δ32 and Δ38 proteins contain relatively more uncharged residues not found in the other proteins. To determine whether these uncharged residues affect the function of these proteins, charged residues were substituted for hydrophobic amino acid residues in the Δ38 protein (Figure 3). Additionally, the complementing Δ43 protein was altered to contain hydrophobic amino acid residues. The resulting proteins are referred to as Δ38C and Δ43U, respectively, and were assayed for complementation activity as described above. The Δ38C protein supported morphogenesis at elevated temperatures, suggesting that uncharged residues in Δ38 were responsible for the lack of activity. However, the insertion of hydrophobic residues into the Δ43 protein did not abrogate activity. Hence, the simplest model, based solely on the presence or absence of hydrophobic residues in the N terminus, is not supported by the data. The specific positioning of the NH_2-terminal residues appears to be critical as well.

Table 1. Complementation of *och*(*B*)*Q3* by ΔNH_2 internal scaffolding proteins

ΔNH_2 B protein[a]	24 °C	33 °C	37 °C	42 °C
Δ22	R_f[b]	1.0	1.0	1.0
Δ27	R_f	1.0	1.0	1.0
Δ32	R_f	R_f	R_f	R_f
Δ38	R_f	R_f	R_f	R_f
Δ43	R_f	1.0	1.0	1.0
Δ53	R_f	1.0	1.0	1.0
Δ58	R_f	R_f	R_f	R_f
Modified proteins[c]				
Δ38C	R_f	R_f	R_f	0.4
Δ43U	R_f	1.0	1.0	1.0

[a] ΔNH_2 B genes are under lac induction in BAF30 (*recA*) cells.
[b] The efficiencies of plating (eop) were determined by comparing the assay titers to the titer determined on cells harboring an expressed clone of the full-length gene. Eop's of one were assigned when titers did not differ more than 10%. R_f represents values equal to the reversion frequency of the ochre mutation (1.0×10^{-6}) as determined on C122 (*sup*°).
[c] See the text for description.

Analysis of the ΔB protein-mediated assembly

To determine the morphogenetic defects associated with the ΔB proteins, the particles synthesized in *och*(*B*)*Q3* infected cells expressing ΔB protein were characterized as described in Materials and Methods. Upon chemical lysis of the infected cells, soluble proteins with densities between 1.3–1.4 ρ were isolated by buoyant density CsCl gradient. Viral particles (virions, procapsids and 70 S particles) were then purified by a second separation in a 5–30% sucrose gradient. After fractionation, the optical density (280 nm) and infectious titer of each fraction was determined.

The sedimentation profiles of particles assembled by Δ38 and wild-type B proteins are presented in Figure 4A. As expected, infectious particles, which sediment at 114 S, were produced in the presence of the wild-type internal scaffolding protein. Although a small 114 S peak of infectious material was also detected in the Δ38 lysates, these virions have lost the ochre phenotype and hence represent background particles produced by *och*(*B*)*Q3* revertants. The major product of Δ38 mediated assembly sediments at 70 S. Defective 70 S particles are off-pathway products that occur after procapsid formation. Despite eops (efficiencies of plating) of $<10^{-6}$ in the plating assays (Table 1), Δ38 B proteins do retain some internal scaffolding functions. This represents the first description of a B^- phenotype

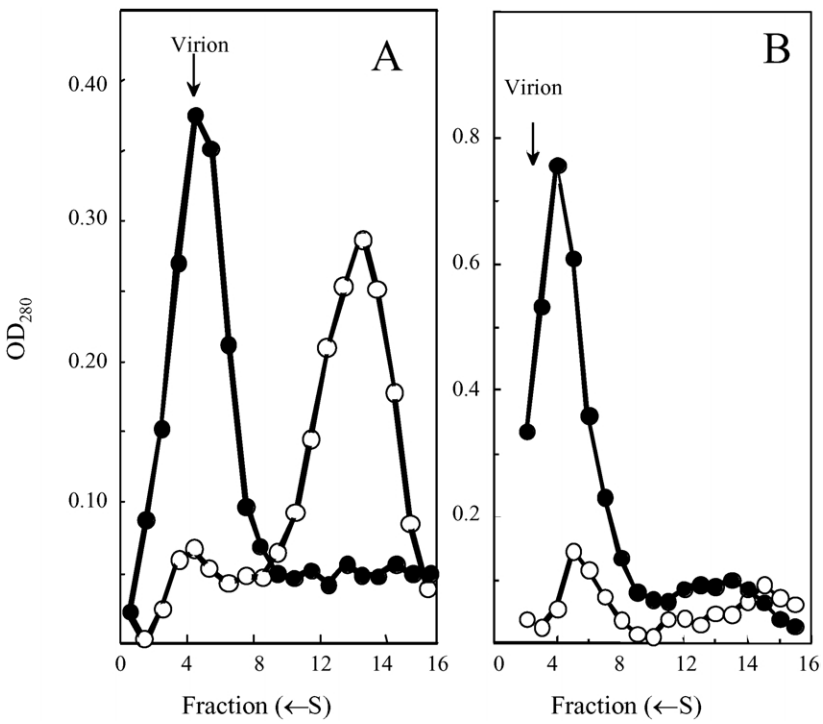

Figure 4. Sedimentation profiles of *och*(*B*)*Q3* particles produced in cells expressing ΔB proteins. After fractionation, fractions were titered for infectious virions and aligned relative to the infectious peaks. In cases where no infectious particles were expected, ϕX174 *am*(*E*) phage were used as a marker and gradients were aligned (A). Particles produced in cells expressing the full length (filled circles) or Δ38 (open circles) protein. B, Particles produced in cells expressing the Δ53 protein at permissive (filled circles, 37 °C) and restrictive (open circles, 27 °C) temperatures.

that supports large particle formation. All previously described internal scaffolding mutants were unable to support the first B-mediated step of assembly, the formation of 12 S particles (Figure 1).[3,15,16]

There are two known mechanisms by which 70 S particles become the dominant particle produced in infected cells (Figure 1). Unfilled procapsids, due to the inability to interact with the DNA packaging machinery, can lose external scaffolding[17] or particles with reduced amounts of protein H, the minor spike protein, extrude portions of the packaged DNA, presumably from 5-fold vertices. This initially yields 110 S particles. Upon subsequent loss of the external scaffolding protein, particles sediment at 70 S.[10] To distinguish between these two possibilities, the protein composition of Δ38 70 S particles was examined by PAGE and compared to virions (Figure 5). In both samples, bands representing the three large structural proteins: coat (F), spike (G), and minor spike protein (H), were present. The band intensities of each sample were analyzed to determine the relative stoichiometry of the proteins (Table 2). While the major spike:major coat protein ratios for each sample are identical, the results of the analyses indicate only 2–3 copies of minor spike protein H per mutant capsid as compared to the 10–12 typically observed in wild-type particles.[10] Although this is the first documentation of a minor spike protein incorporation mutant in the *Microviridae*, recent structural data[9] indicate that the B and H proteins may contact each other in the procapsid (see Discussion).

The particles produced by Δ53-mediated morphogenesis at both permissive and non-permissive temperatures were also characterized. Although infectious virions (114 S) were produced above the background level of ochre reversion, the dominant species produced in these cells sediment between 108–110 S and are not infectious (Figure 4B). These results suggest that particles formed by the Δ53B protein, like the Δ38 B protein, presumably contain less H protein. However, a rigorous analysis of the particles in this region could not be conducted, due to the proximity of

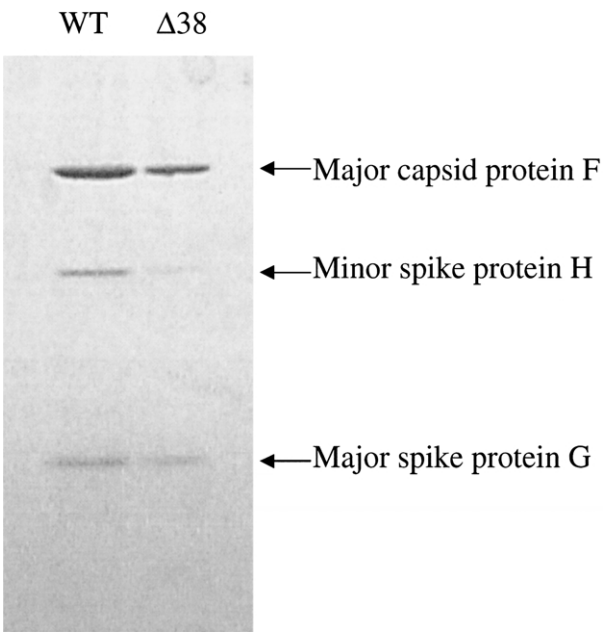

Figure 5. Polyacrylamide gel formed by full length and Δ38 internal scaffolding proteins.

Table 2. Stoichiometric analysis of structural proteins found in wild-type and Δ38 B particles

	Band intensity ratios[a]		Protein ratios[b]	
Proteins	Wild-type	Δ38	Wild-type	Δ38
Major spike:major capsid	0.23	0.20	60:60	52:60
Minor spike:major capsid	0.23	0.06	10–12:60	2.6–3.1:60
Minor spike:major spike	0.93	0.28	10–12:60	3.0–3.6:60

[a] Proteins were separated by SDS-PAGE, stained and digitally photographed. Ratios were calculated using band intensities derived *via* 1D image analysis software (Kodak Digital Science™).
[b] Established wild-type ratios[10] were used to calculate protein ratios for the Δ38 particles.

this peak to infectious virions (114 S) and procapsids (108 S), which are located in adjacent fractions. If there is an H-incorporation defect associated with this Δ protein it is obviously less severe than that associated with Δ38 since infectious particles are produced. At restrictive temperatures, no large particles were detected in extracts from infected cells. These data suggest that the deletion proteins confer pleiotropic defects. Experimentally, the defect in procapsid formation would be epistatic to H protein incorporation. Similar results were obtained in the experiments conducted with the Δ43 at restrictive temperature, and the Δ58B and Δ67 B proteins, which do not support morphogenesis at any temperature.

Second-site genetic analyses

To further explore the molecular basis of the ΔB protein defects, second site mutations, or Δ*B-utilizers*, which restore viability, were isolated as described in Materials and Methods. Although no *utilizers* of the Δ38 or Δ58 proteins were recovered, *utilizers* of Δ43 and Δ53 proteins were readily isolated. Sequencing analyses mapped all Δ*B-utilizers* (8) to one of two amino acid residues of the external scaffolding protein. Each substitution (Thr → Ile at amino acid 46 and Asp → His at amino acid 47) was independently isolated at least two times. The entire genome for one of the mutants was sequenced and no other mutations were found. These suppressors exhibited some allele specificity. They suppress the cold-sensitive phenotypes associated with the Δ22, Δ27, Δ43 and Δ53 proteins, suggesting that the defects associated with these proteins are mechanistically similar. However, they do not suppress the defects associated with the Δ32, Δ38, Δ58, or Δ67 proteins. The extragenic suppressors confer no additional phenotypes other than suppressing the cold-sensitive viability of the ΔB proteins. A subset of this data is presented in Table 3. The region of the external scaffolding proteins in which the *utilizers* reside participates in intra-dimer contacts (D1–D2, D3–D4), inter-dimer contacts within an asymmetric unit (D2–D3), and 5-fold related inter-dimer contacts (D2–D4). Since these alterations in the external scaffolding protein lattice are located in contact regions, these second-site mutations most likely compensate for the inability of the ΔB proteins to fully induce conformational switches in coat protein pentamers.

Discussion

Bacteriophage ϕX174 utilizes two scaffold proteins during morphogenesis, which differs from most larger dsDNA viruses that utilize only one.[18–20] The internal scaffolding (B) and external scaffolding (D) proteins perform all the functions associated with the larger scaffolding proteins found in one scaffolding protein systems.[1] This evolutionary separation of functional domains into two distinct proteins facilitates the depth to which functions performed exclusively by one of the proteins can be studied. However, functions requiring both proteins may be more difficult to dissect due to inherent redundancies in function.

Conformational switching

Early in morphogenesis, the internal scaffolding protein binds to the underside of the coat pentamer, inducing a conformational change, which enables it to interact with spike protein and external scaffolding proteins. The inability of the deletion proteins to mediate procapsid formation at restrictive temperatures suggests that the N terminus is required for efficient conformational switching. While it is possible that the deletion proteins are unable to bind coat protein pentamers

Table 3. Complementation of *och*(*B*)*Q3*/Δ*B-utilizer* strains by ΔB genes

Mutant	Δ38	Δ43	Δ53	Δ58	Wt gene	No gene
och(*B*)*Q3*	10^{-6}	10^{-6}	10^{-6}	10^{-6}	1.0	10^{-6}
och(*B*)*Q3*/Δ*B-ut*(*D*)*T46I*	10^{-6}	0.5	0.3	10^{-6}	1.0	10^{-6}
och(*B*)*Q3*/Δ*B-ut*(*D*)*D47H*	10^{-6}	0.6	0.9	10^{-6}	1.0	10^{-6}

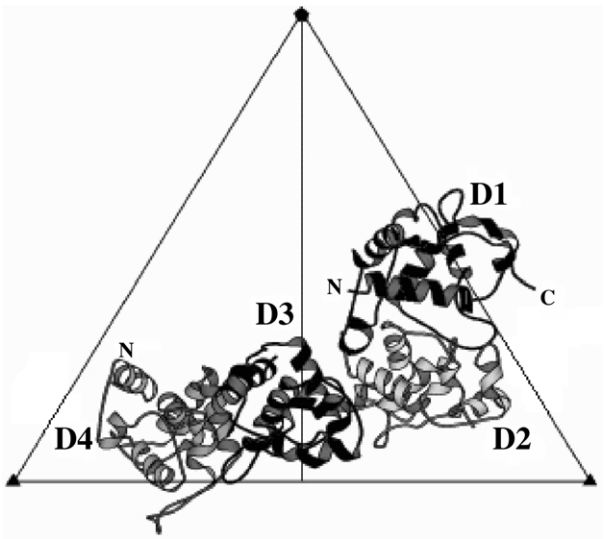

Figure 6. The atomic structure of the external scaffolding protein.

at restrictive conditions, two experimental observations argue against this hypothesis. First, 9 S coat pentamers self-associate when not bound by the internal scaffolding protein, resulting in aggregates sedimenting between 55–65 S. These aggregates were not observed. Secondly, the Δ*B-utilizer* mutations are located in the external scaffolding protein. However they do not circumvent the requirement for the internal protein.

The Δ*B-utilizer* mutations only suppress the defects associated with the deletion proteins of less than 53 amino acid residues and do not actively inhibit minor spike protein incorporation (see below). All of the Δ*B-utilizer* mutations mapped to a specific region of the external scaffolding protein. In the procapsid atomic structure (Figure 6), each icosahedral asymmetric unit contains four copies of the external scaffolding protein (D1–D4), which are arranged as two similar, but not identical, asymmetric dimers.[5,11] The domain containing the *B-utilizer* substitutions participates in intradimer contacts (D1–D2, D3–D4) and interdimer contacts both within the asymmetric unit (D2–D3) and across 5-fold axes of symmetry (D2–D4). The suppressing amino acid residues do not participate in D–D associations across the 2-fold axes of symmetry, which stabilize the procapsid, and there are no 3-fold related D contacts known to exist in the structure. Therefore, the effects of the suppressors would be confined to one pentamer and most likely compensate for the defect of incomplete conformational switching defects associated with the ΔB protein suggesting some inherent redundancy of function between the two scaffolding proteins.

Incorporation of minor spike protein

Early in ϕX174 morphogenesis, minor spike protein H is incorporated into the developing particles. However, large particle formation does not require protein H incorporation. Without H protein, 110 S particles that degrade to 70 S particles are formed.[10] While all of the ΔB proteins may lack the ability to optimally perform H protein incorporation, as evinced by the formation of 110 S particles, the Δ38B protein appears to be particularly defective, as if inhibitory. A high-resolution cryo-image reconstruction (15 Å) of a *Microviridae* procapsid suggests that a portion of the internal scaffolding protein, including the hydrophobic residues from 32 to 38, may be associated with the minor spike protein (H) at the 5-fold axes of symmetry.[9] Site-directed mutagenesis of the Δ38B protein to a more charged state resulted in restored viability at high temperatures. Presumably, H incorporation was restored to some degree. From the high-resolution cryo-image reconstruction and crystallographic data, it is this hydrophobic region of protein B that appears to merge with H protein density underneath the 5-fold axes of symmetry (Figure 2). With the full-length B protein, this hydrophobic region may create a local hydrophobic channel, in an otherwise hydrophilic interior environment of the capsid, facilitating the incorporation of the hydrophobic H protein. Lack of H incorporation, as observed with Δ38 B protein could be attributed to the improper orientation of the B hydrophobic regions due to the absence of hydrophilic amino acid residues directly downstream. Without this NH_2-terminal "anchoring" effect, the five hydrophobic regions could drift inward to interact with each other, thereby inhibiting proper morphogenesis. The P22 scaffolding has been demonstrated to play a direct role in spike protein incorporation.[21] However, in the ϕX174 system, the amino terminus of protein B is not essential for H protein incorporation, but appears to increase efficiency, suggesting a redundancy of function with the major spike and/or coat proteins.

The evolution of essential proteins encoded in overlapping reading frames

Within the ϕX174 genome, gene B lies completely within the coding region of gene A. Protein A must interact with host cell DNA replication proteins, most notably the Rep helicase.[22] Therefore, this protein must evolve within the host to ensure that proper structural interactions are maintained.[7,23] While the other proteins encoded with the coding region of gene A are unessential for viability: proteins A* and K,[24,25] the B protein is essential. However, the protein has evolved characteristics to ensure that the evolution of the virus is not constrained. For example, it is extremely tolerant of missense substitutions. *Microviridae* internal scaffolding proteins cross-function despite only 30% similarity,[12] suggesting that the coat-scaffolding interactions are mediated more by interacting domains than specific amino acid residues. More critical amino acid residues for

coat protein binding, such as those found in the C terminus, appear to be duplicated, being located directly next to an amino acid with a similar side-chain. Furthermore, B-protein cleavage may be required for extrusion from the procapsid.[9] Although the protein is cleaved only once, there are three possible cleavage sites. No site appears to be preferentially utilized, at least *in vitro*. Should a mutation in gene A eliminate one of these cleavage sites, two additional ones remain, and morphogenesis will not be deterred.[26]

The functions of the internal scaffolding protein are also well integrated with the functions of the other viral proteins. This seems to be especially apparent for the N terminus. Deletions eliminating 44% of the protein (Δ53) are functional enough to produce infectious virions, albeit not as efficiently as the wild-type protein. While these proteins demonstrate defects in inducing conformational switches in coat protein pentamers and minor spike protein incorporation, the phage can still replicate. Mutations in the external scaffolding protein can suppress the conformational switching defects. This functional redundancy between the two scaffolding proteins may be an evolutionary safeguard; both are encoded in overlapping reading frames. A similar redundancy may be occurring with the coat and major spike proteins for the incorporation of minor spike protein H. While the amino terminus B protein appears to facilitate its incorporation, it is not strictly required.

Materials and Methods

Phage plating, media, stock preparation, and DNA isolation

The reagents, media, buffers, and protocols have been described.[16]

Bacterial strains and ϕX174 mutants

The *Escherichia coli* C strains C122 (*sup*°), BAF8 (*supF* derivative of *E. coli* C122), BAF9, and BAF30 *recA* have been described.[12,16,27] The C900 *slyD* host confers resistance to ϕX174 E protein-mediated lysis.[28]

The protocol used for oligonucleotide-mediated mutagenesis in the construction of ϕX174 *och*(*B*)*Q3* mutant has been described.[29] The ϕX174 *am(B)Q18* mutant has been described.[16]

Generation of cloned B genes with deletions of the amino terminus

The clones of the ΔB genes were generated by PCR. The upstream primer mutagenized the viral DNA to insert both an ATG start codon as well as an *Nco*I restriction site at the 5′ end of the Δ gene. A second primer was located downstream from the ϕX174 *Xho*I site (position 162). The PCR fragment was cloned into the TOPO 2.1 vector (Invitrogen). Plasmid DNA was then digested with *Nco*I and *Xho*I then subcloned into pSE420 digested, behind a lac inducible promoter, with the same enzymes. Standard conditions were used for digestion and ligation reactions.[30] All clones are under lactose induction. All ΔB genes were verified by restriction digestion and sequence analyses. DNA sequencing was performed at the University of Arizona sequencing facility.

Characterization of particles generated in ΔB infections

One liter of *slyD* cells harboring a ΔB plasmid was infected with ϕX174 *och*(*B*)*Q3* (moi = 3) and incubated for 4–6 hours. Infected cells were concentrated by centrifugation and resuspended in 8 ml BE buffer. Cells were lysed with phage T4 lysozyme at 0.8 mg/ml and treated with RNase (0.8 mg/ml). Multiple centrifugations were done at 5 K for 20 minutes to remove cellular debris. Soluble protein and viral particles were purified by buoyant density sedimentation as described.[16] Material from 1.3 to 1.4 ρ; soluble protein-virion, respectively; was removed with a syringe and dialyzed overnight in BE for the second purification by rate zonal centrifugation in sucrose gradients (5–30%) as described,[7] except centrifugation was at 18 °C. Gradients were fractionated and analyzed for large protein particles *via* UV spectrophotometer (A_{280}). Proteins generated from Δ38 B complementation were further analyzed *via* 15% (w/v) polyacrylamide gel electrophoresis.

Isolation of *Δ43/Δ53-utilizers*

1.0×10^8 plaque-forming units of ϕX *och*(*B*)*Q3* were plated on BAF30 pBΔ43 and BAF30pBΔ53 and incubated at 28 °C. Revertant plaques were stabbed into three indicator lawns: C122 (*sup*°), BAF9, and BAF30pϕXB. Putative mutants were identified by the retention of the ochre phenotype and B gene complementation dependent growth. The retention of the parental ochre mutation was also verified by direct sequence analysis.

Acknowledgements

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