Effects of an Early Conformational Switch Defect during ϕ X174 Morphogenesis Are Belatedly Manifested Late in the Assembly Pathway

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C-terminal, aromatic amino acids in the ϕ X174 internal scaffolding protein B mediate conformational switches in the viral coat protein. These switches direct the coat protein through early assembly. In addition to the aromatic amino acids, two acidic residues, D111 and E113, form salt bridges with basic, coat protein side chains. Although salt bridge formation did not appear to be critical for assembly, the substitution of an aromatic amino acid for D111 produced a lethal phenotype. This side chain is uniquely oriented toward the center of the coat-scaffolding binding pocket, which is heavily dominated by aromatic ring-ring interactions. Thus, the D111Y substitution may restructure pocket contacts. Previously characterized B^- mutants blocked assembly before procapsid formation. However, the D111Y mutant produced an assembled particle, which contained the structural and external scaffolding proteins but lacked protein B and DNA. A suppressor within the external scaffolding protein, which mediates the later stages of particle morphogenesis, restored viability. The unique formation of a postprocapsid particle and the novel suppressor may be indicative of a novel B protein function. However, genetic data suggest that the particle represents the delayed manifestation of an early assembly error. This seemingly late-acting defect was rescued by previously characterized suppressors of early, preprocapsid, B^- assembly mutations, which act on the level of coat protein flexibility. Likewise, the newly isolated suppressor in the external scaffolding protein also exhibited a global suppressing phenotype. Thus, the off-pathway product isolated from infected cells may not accurately reflect the temporal nature of the initial defect.

Scaffolding proteins mediate the conformational switches in viral coat proteins that control proper morphogenesis. These switches reduce the thermodynamic barriers in the productive pathway relative to those that promote off-pathway reactions (1–3). Since many assembly systems involve the rapid addition of small elongation units to short-lived nucleation complexes, morphogenesis is typically divided into three stages: coat protein binding, nucleation, and elongation (1–3). While the absence of coat protein binding can be easily distinguished from other defects (4–6), the fluid nature of assembly can obscure the differences between defects in the later stages. An altered nucleation complex could result in the formation of an aberrantly shaped assembled particle (4, 7).

Unlike most assembly systems, ϕ X174 morphogenesis is mediated by two scaffolding proteins, an internal and an external species. These proteins temporally divide the pathway into two discernible phases (Fig. 1A). During early morphogenesis, five copies of the internal scaffolding protein B bind to the underside of the 9S coat protein F pentamer forming the 9S* particle (8). This induces the conformational changes that (i) inhibit 9S particle aggregation, (ii) facilitate DNA pilot protein H incorporation, and (iii) stimulate coat-spike protein interactions (8–12). During late morphogenesis, 240 copies of the external scaffolding protein D organize 12 12S* particles into procapsids (13). As the genome is packaged, the DNA binding protein J displaces the internal scaffolding protein. Extrusion is most likely facilitated by the B protein's autoproteolytic activity (14). The resulting metastable provirion is infectious (15–17). Spontaneous dissociation of the external scaffolding protein yields the mature virion.

As seen with the P22, P2, ϕ 29, and herpes simplex virus (HSV) internal scaffolding proteins (18–22), the C terminus of the ϕ X174 B protein is most strongly associated with the coat protein

(23–25). In the ϕ X174 procapsid crystal structure, six aromatic amino acid residues mediate approximately 85% (22/26) of these interactions. Most of the participating atoms (17/22) are found in aromatic rings (Fig. 1B). The results of a previous genetic analysis demonstrate that nonaromatic substitutions for residue F120, the last amino acid, or the absence of this amino acid, abolish coat protein binding (5). Substitutions at the other aromatic sites produced defective conformational switching phenotypes. The mutant proteins kinetically trapped early assembly intermediates after coat protein binding, the assembly phase mediated by the internal scaffolding protein, but prior to procapsid formation, which is mediated by the external scaffolding protein. Although the internal scaffolding protein mutations trap different intermediates within the first half of the assembly pathway, common suppressors alleviate the various morphogenetic defects. This suggests a fluid assembly pathway, one in which the scaffolding protein induces a single, coat protein conformational switch, not a series of sequential reactions. In this model, the nature of the improper switch determines the trapped intermediate. Consequently, neither a specific function nor a temporal requirement could be assigned to any particular amino acid.

In addition to the C-terminal aromatic side chains, two acidic residues, D111 and E113, form salt bridges with coat protein amino acids R239 and K166 (Fig. 1B). Electrostatic interactions

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FIG 1 ϕ X174 morphogenesis and the structures of the coat and scaffolding proteins. (A) Wild-type and *l(B)D111Y* assembly pathways. The 9S* intermediate contains the coat F, internal scaffolding B, and minor vertex H proteins in a respective 5:5:1 ratio. The addition of the major spike protein G pentamer (6S particle) produces the 12S* particle. A total of 240 copies of the external scaffolding D protein organize 12 12S* particles into procapsids. (B) Structure of the coat-internal scaffolding protein binding cleft with the ϕ X174 procapsid crystal structure (Protein Data Bank code, 1C3D). Labels depict the protein (F, coat; B, internal scaffolding), amino acid (letter), and position (number) in the primary structure. The three coat protein aromatic amino acid residues—F-F67, F-Y134, and F-F135—that participate in ring-ring contacts with internal scaffolding protein residue B-F120 are highlighted in lavender. (C) Atomic structure of the coat (gray) and internal scaffolding (peach) proteins. (D) Locations of second-site suppressors of internal scaffolding (peach) and external scaffolding (teal) protein defects. The positions of the newly isolated suppressors at amino acids 203 and 314 are highlighted.

govern morphogenesis in other viral systems (3) and have been thoroughly studied in bacteriophage P22 (4, 6, 26–28). In the P22 scaffolding protein, two charged residues are central to coat protein binding, whereas other amino acids modulate activity by promoting the proper architecture of the coat-scaffolding protein interface (4, 26). Alteration of an amino acid that mediates a protein-protein interaction, either via a salt bridge or via an aromatic ring, is typically regarded as a loss-of-function, or loss-of-contact, mutation. The previously characterized mutations at aromatic sites most likely fall into this category (5). In contrast, the introduction of aromatic residue into an environment already rich in aromatic content could create a gain-of-contact mutation,

which may compete with wild-type interactions. To investigate whether charged amino acids are critical to ϕ X174 B protein function, as seen in the P22 scaffolding protein, or play a more subsidiary role, aromatic and nonaromatic substitutions for D111 and E113 were genetically and biochemically characterized.

MATERIALS AND METHODS

Phage plating, media, buffers, and stock preparation. The reagents, media, buffers, and protocols have been previously described (29).

Bacterial strains, phage strains, and plasmids. The wild-type *Escherichia coli* C strain C122 (Su[−]) and the isogenic, amber-suppressing strains BAF7 (*supD*), BAF5 (*supE*), BAF8 (*supF*), and BAF30 (*recA*) have

been previously described (29, 30). The host *slyD* mutation confers resistance to E protein-mediated lysis (31). The construction of the complementing plasmid p ϕ XB has been previously reported (5).

Generation of *am(B)* and missense *B*[−] mutations. Unless otherwise indicated, amber and missense mutants were generated by oligonucleotide-mediated mutagenesis utilizing the published protocol (32). Mutagenized DNA was transfected in BAF30 p ϕ XB, which expresses the wild-type B gene. Mutants were identified by complementation-dependent phenotypes. Genotypes were verified by a direct DNA sequence analysis.

Isolation and construction of *am(B)* and missense *B*[−] mutations, second-site suppressors, *su/am* strains used in the cross-suppressor analysis, and *su(B111)-D V75A l(B)D111Y* mutant. All protocols were identical to those described in an earlier publication (5), with the exception of *su(B111)-D V75A l(B)D111Y* strain construction, which was made by recombination rescue. The *l(B)D111Y* gene was amplified and cloned as previously described (33). To select for *am*⁺ recombinants, the *su(B111)-D V75A am(B)D111* mutant was plated on cells harboring the clone gene, which was not induced. All genotypes were verified by a direct sequence analysis.

Detection of virion, intermediate particles, and DNA. To generate extracts of infected cells, 100 ml of lysis-resistant cells at 1.0×10^8 cells/ml were infected with wild-type or mutant ϕ X174 at a multiplicity of infection (MOI) of 5.0 as previously described (34). Subsequent extract preparation, rate zonal sedimentation, and protein electrophoresis protocols have already been published (34). For the detection of particles with S values larger than 70, samples were loaded atop 5 to 30% sucrose gradients and spun at 45,000 rpm for 1.0 h in a Beckman SW50.1 rotor. After fractionation into approximately 50 100- μ l fractions, virally derived protein complexes were detected by UV spectroscopy ($\lambda = 280$ nm). The position of infectious virions (114S) was further determined by plating assays. To detect particles with S values of less than 70, samples were spun at 34,000 rpm for 16 h. After fractionation, material was detected by 15% SDS-PAGE. Modified 15 to 30% sucrose gradients were prepared by the addition of CsCl (3.5%, wt/vol) to the 30% sucrose layer before mixing (13). DNA was extracted directly from particles isolated from sucrose gradient fractions using established protocols (29).

RESULTS

The introduction of a C-terminal aromatic amino acid results in a lethal phenotype. Internal scaffolding protein residues D111 and E113 form respective salt bridges with coat protein residues R239 and K166. To determine whether these interactions were critical to B protein function, amber (*am*) mutations were generated in these codons. Mutants were assayed for plaque formation on three tRNA informational suppressing hosts (*Su*⁺), which insert glutamine, serine, or tyrosine at the amber codon during protein synthesis (29). Thus, a single nonsense mutation can be used to generate a variety of missense proteins. As determined in plaque assays on the various tRNA informational suppressing hosts, viability serves as an indicator of protein function, assessing the consequences of altering the wild-type interactions. All three amino acid substitutions for E113 were tolerated, suggesting that the E113-K166 electrostatic interaction is not essential (Table 1). Serine and glutamine, which would either eliminate or substantially alter the electrostatic D111-R239 interaction, were also tolerated, albeit poorly. However, there was a strong lethal phenotype associated with the introduction of an aromatic tyrosine residue.

The D $\rightarrow$ S and D $\rightarrow$ Q substitutions at position 111 would alter the D111-R239 salt bridge and can be regarded as a partial loss of function. Although the D $\rightarrow$ Y substitution would also eliminate this interaction, the consequences of this substitution appeared to be much more severe (Table 1). Considering the numerous aromatic ring-ring interactions between the coat and internal scaffold-

ing protein, the effects of D $\rightarrow$ Y substitution may be more complex. At the center of the coat-scaffolding protein binding cleft, scaffolding protein residue F120 participates in aromatic ring-ring interactions with coat protein residues F67, Y134, and F135 (Fig. 1B). Altering these contacts strongly inhibits coat-scaffolding protein interactions (5). Considering the proximity and orientation of the D111 side chain toward the cleft's center, the introduction of an aromatic amino acid at this position may abrogate coat-internal scaffolding protein interactions, which would lead to the intracellular coat protein aggregation (5), or introduce a novel interaction that redirects assembly. To distinguish between these two hypotheses, the morphogenetic pathway was examined in missense mutant-infected cells.

The D111Y substitution produces an off-pathway procapsid-like particle. The inefficiency of informational suppression as well as defective protein function can contribute to amber mutant phenotypes in *Su*⁺ hosts (35, 36). Therefore, a D $\rightarrow$ Y missense mutation was generated directly in the viral genome. The resulting *l(B)D111Y* mutant exhibited a lethal phenotype but was rescued by the exogenous expression of a wild-type B gene. Thus, defective protein function is the primary phenotypic determinant. To assay the mutant protein's effect on particle morphogenesis, lysis-resistant cells were infected with the *l(B)D111Y* mutant or wild-type ϕ X174 as described in Materials and Methods. Infected-cell extracts were generated and analyzed by rate zonal sedimentation with 5 to 30% sucrose gradients. In both extracts, particles sedimenting between 108S and 114S, procapsids and virions, were detected (Fig. 2A). However, the specific infectivity (PFU/A₂₈₀) of the *l(B)D111Y* mutant peak was approximately 2 orders of magnitude lower than that of the wild-type control (Table 2). In addition, degraded procapsids (70S particles) were prominent in the *l(B)D111Y* extract. The D111Y protein appeared to redirect assembly. All previously isolated internal scaffolding protein mutations arrest assembly before procapsid assembly. Thus, the molecular phenotype of this mutation is unique.

Particle protein composition was examined by SDS-PAGE (Fig. 3A). As the coat protein F remains soluble in the *l(B)D111Y* extracts, the D111Y mutation did not block coat protein binding (5). The wild-type material from 108S to 114S contained both scaffolding proteins, proteins B and D, indicating a mixture of procapsids and virions. In contrast, the mutant particles from 108S to 114S lacked protein B, the internal scaffolding protein. This protein composition resembles wild-type provirions (Fig. 1A). However, provirions are infectious and sediment more quickly at 132S to 140S (16). The mutant 70S material lacked both scaffolding proteins. In a wild-type infection, 70S degraded procapsids result from the loss of the external scaffolding protein D. However, they retain the B protein (37, 38).

To determine whether the *l(B)D111Y* particles represent kinetically trapped provirions or a novel off-pathway product, their sedimentation properties were analyzed in 15 to 30% sucrose gradients modified by the addition of CsCl (13), which more accurately separates procapsids (108S) from DNA-filled provirions (132S). The provirion position within the wild-type control gradient was determined by identifying the fastest-sedimenting material with a specific infectivity equal to the prominent, mature virion peak (Table 2). Again, *l(B)D111Y* particles appear to sediment more slowly than wild-type virions (Fig. 2B) and are less infectious (Table 2). Wild-type and second-site revertants constitute the background signal in specific-infectivity assays. All viable

TABLE 1 Phenotypes of missense suppressed *am(B)* and *su/am(B)* strains^a

Genotype of strain	Defect associated with missense protein ^b	<i>am</i> strains				<i>su/am</i> strains				
		Phenotype with insertion of aa ^c :				Suppressor mutation	Phenotype with insertion of aa:			
		Q	S	Y	RF(<i>am</i> ⁺) ^d		Q	S	Y	RF(<i>am</i> ⁺)
<i>am(B)D111</i>	See the text.	10 ⁻²	1.0	RF	10 ⁻⁴	<i>su(B111)-B F109L</i>	0.7	0.7	0.8	<10 ⁻⁵
						<i>su(B111)-BN115D</i>	1.0	1.0	1.0	10 ⁻³
						<i>su(B111)-D V75A</i>	10 ⁻²	10 ⁻²	10⁻¹	<10 ⁻⁴
						<i>su(B111)-F H203Y</i>	1.0	0.9	1.0	10 ⁻⁵
						<i>su(B111)-F A314V</i>	1.0	1.0	1.0	10 ⁻⁴
						<i>su(B106)-F P325S</i>	0.8	0.8	0.9	<10 ⁻⁴
						<i>su(B107)-F R382C</i>	0.8	1.0	1.0	<10 ⁻⁴
						<i>su(B117)-F T178I</i>	RF	RF	RF	10 ⁻⁴
<i>am(B)F106</i>	F106Y: 9S*→12S* transition	RF	RF	RF	10 ⁻³	<i>su(B111)-D V75A</i>	RF	RF	1.0	10 ⁻²
	12S* particle produced in reduced quantity					<i>su(B111)-F H203Y</i>	RF	RF	1.0	10 ⁻⁵
<i>am(B)Y107</i>	Y107S: B protein loss from 9S* particle	RF	RF	1.0	10 ⁻⁷	<i>su(B111)-D V75A</i>	1.0	1.0	1.0	10 ⁻⁵
						<i>su(B111)-F H203Y</i>	RF	RF	1.0	10 ⁻⁶
<i>am(B)F109</i>	F109Q: uncharacterized	RF	RF	0.7	10 ⁻⁶	<i>su(B111)-D V75A</i>	1.0	1.0	1.0	10 ⁻⁵
						<i>su(B111)-F H203Y</i>	RF	RF	1.0	10 ⁻⁵
<i>am(B)F117</i>	F117Q: uncharacterized	RF	RF	1.0	10 ⁻⁵	<i>su(B111)-D V75A</i>	0.7	RF	1.0	10 ⁻⁵
						<i>su(B111)-F H203Y</i>	RF	RF	1.0	10 ⁻⁴
<i>am(B)Y119</i>	Y119W: 12S*→procapsid transition. The phenotype associated with nonaromatic substitutions is presumed to affect coat protein binding (5).	RF	RF	1.0	10 ⁻⁵	<i>su(B111)-D V75A</i>	RF	RF	1.0	10 ⁻⁴
						<i>su(B111)-F H203Y</i>	RF	RF	1.0	10 ⁻⁶
<i>am(B)F120</i>	F120K: coat protein binding	RF	RF	0.6	10 ⁻⁶	<i>su(B111)-D V75A</i>	RF	RF	1.0	10 ⁻⁶
						<i>su(B111)-F H203Y</i>	RF	RF	1.0	10 ⁻⁶
<i>am(B)E113</i>	None	0.8	0.7	1.0	10 ⁻⁴					

^a Data are presented as efficiency of plating: assay titer/most permissive titer. The most permissive titer was determined on either BAF 8 *supF*, which inserts tyrosine during protein synthesis at amber codons, or BAF30 pΦXB, which expresses a cloned wild-type B gene. Boldface indicates the conditions under which rescue by the suppressor occurred. The names of the amber mutants reflect the wild-type codon and encoded amino acid. Thus, *am(B)D111* indicates an amber mutation in codon 111 that normally encodes an aspartic acid (D). The names of the second-site suppressors reflect the parental background in which they were isolated, the gene in which the suppressor resides, and the resulting substitution. Thus, *su(B111)-D V75A* indicates that the suppressor was originally isolated in the *am(B)D111* background. “-D” indicates an amino acid substitution in the external scaffolding protein D; “V75A” indicates a valine (V)→alanine (A) substitution. aa, amino acid.

^b The column summarizes the data published by Gordon et al. (5). Biochemical analysis was conducted with genome-encoded missense mutants. The missense substitution is listed before the colon, the molecular phenotype is described after the colon. In some instances, the defective phenotypes associated with missense mutants were too weak to biochemically characterized. These are identified with the word “uncharacterized.”

^c Letters indicate the amino acid inserted by the tRNA informational suppressor. Q, glutamine; S, serine, Y, tyrosine.

^d RF (*am*⁺), the reversion frequency of the amber mutation as determined on the Su⁻ host.

particles detected within the mutant samples were revertants, which provided a valuable internal S value marker. However, specific infectivity is defined as PFU/*A*₂₈₀, regardless of the genotype of the detected particle. Thus, the reported *l(B)D111Y* specific infectivity is most likely lower than the assay suggested. All fractions were examined by SDS-PAGE. Unlike the wild-type control, the internal scaffolding protein was not detected within any fraction (Fig. 3B).

The *l(B)D111Y* particles are most likely not kinetically trapped provirions, which would be DNA-filled but off-pathway particles. To validate this conclusion, extracts of wild-type- and mutant-infected cells were prepared, assembled particles (≥70S) were separated from unpackaged DNA by sucrose gradient sedimentation, and nucleic acid was extracted (Fig. 3C). Genomic DNA was abundantly associated with the wild-type particles. However, only trace amounts were detected in the mutant sample, which was 20-fold more concentrated than the wild-type control. The DNA recovered from the mutant sample most likely reflects the back-

ground revertants detected in the specific-infectivity assays. Thus, the mutant D111Y internal scaffolding protein prematurely left the assembly pathway before being displaced by the DNA packaging process.

To more accurately gauge when the mutant B protein leaves the assembly pathway, the protein composition of the early, preprocapsid intermediates was determined. Wild-type and mutant 9S and 12S particles were isolated as described in Materials and Methods. Since most viral proteins were associated with large assembled particles, fractions had to be pooled and concentrated 25-fold to gather enough material for the analysis. As can be seen in Fig. 3D, the internal scaffolding protein was not detected in the early assembly intermediates isolated from the *l(B)D111Y* infection, whereas it was present in the wild-type particles. These data indicate that either the mutant protein prematurely left the assembly pathway before procapsid formation or its association with the viral coat protein F was too weak to withstand the purification protocol.

FIG 2 Large particles ($S > 50$) synthesized in cells infected with the wild type and *l(B)D111Y* and *su(B111)-D V75A l(B)D111Y* mutants. (A) Sedimentation (5 to 30% sucrose) of extracts of cells infected with the wild type (○) and *l(B)D111Y* mutant (●). The fraction with the highest specific infectivity is labeled 114S, the sedimentation value of the mature virion. In a wild-type infection, 70S material contains degraded procapsids resulting from the loss of the external scaffolding protein. Lower fractions represent the bottom of the gradient, which would contain faster-sedimenting material. (B) Sedimentation (15 to 30% CsCl-modified

Second-site genetic analysis of the D111→Y substitution yielded novel suppressors. To determine whether viruses could adapt to the mutant internal scaffolding protein, a second-site genetic analysis was conducted. To select for second-site suppressors, *am(B)D111* was plated on the restrictive Su^+ host, BAF8, which inserts tyrosine residues at amber sites during protein synthesis. The resulting plaques were stabbed into Su^+ and Su^- indicator lawns. Putative second-site suppressors were identified by the retention of the amber phenotype. One of the mutations, *su(B111)-F A314V*, was very similar to known coat protein suppressors of conformational switch defects (Table 1). However, *su(B111)-F H203Y* would alter the large coat protein α -helix at the 3-fold axis of symmetry (Fig. 1C and D). Mutations in this helix are known to suppress defective external scaffolding protein function (see Discussion). Two suppressors, the *su(B111)-B F109L* and *N115D* mutations, resided in gene B. Intragenic suppressors have not been previously isolated for B protein defects (5, 29, 39). Finally, the fourth suppressor, the *su(B111)-D V75A* mutation, changes the external scaffolding protein. A full genome sequence was determined for the *su(B111)-D V75A am(B)D111* and *am(B)D111* mutations. With the exception of the suppressor mutation, no other changes were found. Thus, the identified suppressor was both necessary and sufficient to confer the altered phenotype.

The location of the suppressor in the external scaffolding protein was unique. The 11 previously isolated suppressors of C-terminal, B protein mutations were all located in the viral coat protein F (5). After being placed into the *l(B)D111Y* background, the effects of the *su(B111)-D V75A* mutation were examined biochemically as described above. As can be seen in Fig. 2C, particles migrated at two speeds, represented by the 108S to 114S and 70S peaks in the sedimentation profile. The material from 108S to 114S was analyzed by SDS-PAGE. Unlike for the wild-type control, which contained a mixture of virions and procapsids, neither scaffolding protein was detected, suggesting that the fractions primarily contained virions (data not shown). Moreover, the specific infectivity (PFU/ A_{280}) was comparable to that of the wild-type control (Table 2). The 70S particles lacked infectivity. Although the internal scaffolding protein was detected by SDS-PAGE, it appeared to be present at a greatly reduced quantity (data not shown), requiring both silver staining and laser scanning to be visualized. Thus, the suppressor most likely does not act by stabilizing the mutant B protein within the procapsid. To further investigate both the nature of the initial defect and possible suppression mechanisms, the genetic analysis was expanded to include the previously characterized coat protein binding mutations, conformational switching mutations, and their associated suppressors.

Extragenic suppressors of C-terminal B protein substitutions do not exhibit strict allele specificity. With the exception of coat protein binding mutants, previously characterized B proteins with C-terminal mutations exhibited defects in conformational switching, halting assembly before procapsid formation (5), whereas the *l(B)D111Y* mutant generated a postprocapsid, off-

sucrose) of extracts of cells infected with the wild type (○) and *l(B)D111Y* mutant (●). The position of the metastable, infectious provirion (132S to 140S) in the wild-type fractions was estimated as described in the text. (C) Sedimentation (5 to 30% sucrose) of extracts of cells infected with the wild type (○) and *su(B111)-D V75A l(B)D111Y* mutant (●).

TABLE 2 Specific infectivities of mutant and wild-type particles^a

Gradient and strain genotype	S value	Specific infectivity (PFU/A ₂₈₀)	Specific infectivity normalized to WT
5–30% sucrose gradient			
WT	108–114S	5.0×10^{11}	1.0
<i>l(B)D111Y</i>	108–114S	2.0×10^{10}	0.04
<i>su(B111)-D75V</i>	108S–114S	1.0×10^{12}	2.0
<i>l(B)D111Y</i>			
15–30%, CsCl-modified sucrose gradient			
WT	108S–114S	1.0×10^{12}	1.0
	132S	1.2×10^{12}	1.2
<i>l(B)D111Y</i>	108S–114S	1.6×10^{10}	0.016

^a WT, wild type.

pathway product. This difference in phenotype could reflect different molecular defects. Alternatively, the defects could be fundamentally related. If the latter, the second-site suppressors of both the *D111Y* substitution and the previously characterized conformational switching mutants should cross-rescue. To test this hypothesis, a cross-suppressor analysis was performed. Three previously isolated suppressors of C-terminally mutated B proteins (5) were placed into the *am(B)D111* background. In addition, *su(B111)-D V75A* and *su(B111)-F H203Y* were crossed into previously characterized *am(B)* genomes. The coat and internal scaffolding genes were sequenced in every strain. A full genome sequence of the *su(B111)-D V75A am(B)F107* mutant was determined. No additional substitutions were identified.

Growth phenotypes were characterized on the Su^+ hosts (Table 1). Cross-suppressing activity was defined by plaque formation 2 orders of magnitude above the frequency obtained on the parental mutant's restrictive Su^+ host. Two of the previously isolated suppressors of defective B protein function, *su(B106)-F P325S* and *su(B107)-F R382C*, restored viability to the missense suppressed *am(B)D111* mutant. This observation supports our fluid assembly model (5). Although *su(B117)-F T178I* exhibited no activity in this background, it is known to be one of the weaker suppressors (5). The *su(B111)-F H203Y* suppressor was active only in the *am(B)F106* background, which may reflect the later-acting defects associated with substitutions at this site (see Discussion). With the exception of nonaromatic substitutions at sites F120 and Y119, which eliminate coat protein binding, the *su(B111)-D V75A* mutation rescued all missense suppressed *am(B)* mutants.

DISCUSSION

Coat-internal scaffolding salt bridges are less critical than aromatic-amino-acid-mediated interactions. Six C-terminal aromatic amino acids and two acidic residues mediate ϕ X174 coat-internal scaffolding protein interactions (24, 25). Four of the aromatic amino acids—F106, Y107, Y119, and F120—are essential for proper internal scaffolding protein function (5). Substitutions at these sites prevent coat protein binding or fail to fully induce the requisite conformational switches for procapsid assembly. In contrast, the salt bridges involving D111 and E113 appear to be less critical. Although the E113→S, Q, and Y and D111→S and Q substitutions would eliminate or alter the respective salt bridges formed with coat protein residues K166 and R239, they do not eliminate viability.

Thus, in ϕ X174, charged residues do not strongly govern coat-scaffolding interactions as observed in bacteriophage P22 (4, 6, 26–28). The coat protein-binding domain of the P22 scaffolding protein has a helix-turn-helix (HTH) motif (21). The essential charged residues are located in the C-terminal helix. However, mutations within the N-terminal helix and the intervening β -turn can alter assembly kinetics and eliminate viability without grossly disrupting domain structure or protein binding (26). These observations suggest that some P22 residues perform a subsidiary role, optimizing the architecture of the coat-scaffolding interface. Salt bridges appear to play this secondary role in the ϕ X174 pocket, whereas aromatic ring-ring interactions, mediated primarily by F120, govern coat protein binding.

Restructuring the coat-internal scaffolding protein binding pocket. The orientation of the D111 side chain toward the center of the pocket (Fig. 1B) may explain the unique *l(B)D111Y* phenotype. An aromatic amino acid at position 111 may compete with F120's interactions with coat protein residues F67, Y134, and F135. Unlike previously characterized internal scaffolding protein mutations (5, 29, 39), C-terminal intragenic suppressors of the D111Y defect were isolated. This may reflect unique structural rearrangements within the cleft. However, the intragenic suppressors were not crossed into other mutant backgrounds to demonstrate allele specificity. The full extent to which the binding pocket can be restructured to support the entire assembly process remains to be determined. This will require more targeted and exhaustive genetic analyses. First, the D111Y mutation, perhaps with intragenic suppressors, will need to be placed in *cis* with a nonsense or missense mutation in codon F120. Additional genetic selections for viability may also be necessary. As the B gene overlaps with other genes and regulatory sequences, the mutant gene may need to be expressed from a plasmid in a complementation-based system.

FIG 3 Protein and DNA compositions of wild-type (WT) and *l(B)D111Y* particles. (A and B) Protein compositions of large particles. Fractions analyzed by SDS-PAGE, respectively, correspond to the sedimentation profiles in Fig. 2A (5 to 30% sucrose gradients) and Fig. 2B (15 to 30% CsCl-modified sucrose gradients). (C) DNA isolated from wild-type and *l(B)D111Y* particles. ϕ X174 single-stranded DNA (ssDNA) and replicative-form double-stranded DNA (dsDNA) are included as migration references. (D) Protein composition of pooled 9S to 12S fractions, which contain early assembly intermediates, from extracts of cells infected with the wild type and the *l(B)D111Y* mutant. P, purified procapsids.

Late-acting versus global suppressors of B^- conformational switch defects. The *su(B111)-D V75A* mutation may represent a global suppressor of B^- conformational switch defects. It was active in all backgrounds except those that prevent coat protein binding. In this two-scaffolding protein system, the external scaffolding protein may be able to compensate for reduced internal scaffolding protein function. In a previous study (9), progressive and targeted genetic selections were employed to lessen and finally eliminate B protein dependency. The primary adaptations in the sextuple mutant *B-free* strain involved the overexpression of a mutant external scaffolding protein D and strengthening of its contacts with the coat protein. This external scaffolding protein override mechanism most likely differs from the mechanism conferred by the coat protein suppressors (see below).

In contrast, the *su(B111)-F H203Y* mutation, which is located in the large α -helix at the 3-fold axis of symmetry, exhibited some allele specificity. Substitutions in this helix have pleiotropic effects. They suppress mutant external scaffolding proteins that (i) inefficiently promote the $12S^* \rightarrow$ procapsid transition and (ii) fail to stabilize the 3-fold-axis-related, procapsid pores through which genomic DNA is packaged (34, 40, 41). These are both late-acting defects. The *l(B)D111Y* particles appeared to be empty, suggesting that this suppressor may act at the level of pore stabilization. When crossed into other B^- conformational switch-defective backgrounds, it rescued only the defects associated with the F106Y protein. The F106Y substitution confers a temporally later defect: $12S^*$ particles form, albeit it at low efficiency (5). Considering the previously defined role of the affected α -helix, the suppressor may facilitate procapsid formation at reduced $12S^*$ particle concentrations.

Late-acting defects versus belated manifestations and fluid assembly. The *l(B)D111Y* assembled particles may indicate that the D $\rightarrow$ Y substitution confers a unique late-acting defect, indicative of a novel B protein function. Alternatively, the assembled particles may represent the delayed manifestation of an early assembly error. In this model, mutant $12S^*$ -like pentamers interact with the external scaffolding protein but redirect morphogenesis off-pathway (Fig. 1A). Biochemical data favor this interpretation. The D111Y protein was detected in neither early assembly intermediates nor the final off-pathway product. In the complete absence of protein B, wild-type assembly does not continue past 9S and 6S particle formation, which leads to coat protein aggregation (5). Therefore, the D111Y mutant protein bound the 9S particle, induced a conformational switch, but prematurely left the assembly pathway.

The results of the genetic analyses also indicate that the D111Y defect occurred early in assembly pathway, before procapsid formation, but is belatedly manifested after procapsid morphogenesis. Previously characterized conformational switch mutations kinetically trapped assembly intermediates before procapsid formation (5). Although mutations trapped different early intermediates, they were rescued by a set of common suppressors. While the suppressor substitutions are spread throughout the viral coat protein, their nature offered more insights than their spatial location. They primarily involved glycine and proline residues, suggesting that an inherently flexible coat protein compensates for the reduced ability of the internal scaffolding protein to induce a conformational switch. These suppressors also rescued the defects associated with D111Y substitution, which is consistent with a previously described fluid assembly model. In that model, the

scaffolding protein induces a single, coat protein conformational switch, not a series of sequential reactions. Incomplete or improper switches would kinetically trap intermediates or direct intermediates of the productive pathway. But as the molecular characterization of the *l(B)D111Y* mutant may demonstrate, the off-pathway product isolated from infected cells may not accurately reflect the temporal nature of the initial defect.

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