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Uncoupling the functions of a multifunctional protein: The isolation of a DNA pilot protein mutant that affects particle morphogenesis

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ABSTRACT

Defective øX174 H protein-mediated DNA piloting indirectly influences the entire viral lifecycle. Faulty piloting can mask the H protein's other functions or inefficient penetration may be used to explain defects in post-piloting phenomena. For example, optimal synthesis of other viral proteins requires *de novo* H protein biosynthesis. As low protein concentrations affect morphogenesis, protein H's assembly functions remain obscure. An H protein mutant was isolated that allowed morphogenetic effects to be characterized independent of its other functions. The mutant protein aggregates assembly intermediates. Although excess internal scaffolding protein restores capsid assembly, the resulting mutant H protein-containing particles are less infectious. In addition, nonviable phenotypes of *am(H)* mutants in Su^+ hosts, which insert non-wild-type amino acids, do not always correlate with a lack of missense protein function. Phenotypes are highly influenced by host and phage physiology. This phenomenon was unique to *am(H)* mutants, not observed with amber mutants in other genes.

Introduction

While the øX174 H protein's role as a DNA piloting protein was defined over 30 years ago (Jazwinski et al., 1975a; Jazwinski et al., 1975c), a full array of its functions remains to be elucidated. At the onset of infection, protein H pilots incoming DNA to cell wall adhesion regions on the outer membrane (Azuma et al., 1980; Bayer and Starkey, 1972; Jazwinski et al., 1975b). This reaction is most likely mediated by a predicted N-terminal trans-membrane helix (Tusnady and Simon, 2001). However, this has yet to be experimentally demonstrated. From its position associated with the outer membrane, protein H and the single-stranded (ss) DNA genome is delivered to the cytoplasmic membrane, the site of DNA synthesis (Azuma et al., 1980). During stage I DNA synthesis, the infecting ssDNA is converted into a double stranded replicative form (RF) molecule. Due to the positive polarity of the genome, this occurs without *de novo* viral protein synthesis. Although the second stage of DNA synthesis, the amplification of the RF molecule, occurs after viral protein synthesis, *de novo* H protein synthesis is not required for this reaction (Ruboyianes et al., 2009).

Inefficient penetration will affect the entire viral lifecycle. Thus, piloting defects may have masked the identification of other functions in past genetic and biochemical analyses (Spindler and Hayashi, 1979) or inefficient penetration may have been used to explain defects in post-piloting phenomena, such as viral DNA or protein biosynthesis. As assembly requires critical concentrations of structural and scaffolding proteins, suboptimal viral protein levels could affect particle morphogenesis (Fane and Prevelige, 2003; Prevelige et al., 1988; Uchiyama et al., 2007). In order to evaluate post-DNA piloting functions, processes must be uncoupled. It was recently shown that the optimal synthesis of other viral proteins requires *de novo* H protein biosynthesis, a post-penetration and DNA replication phenomenon (Ruboyianes et al., 2009). Wild-type (Su^-) cells infected with permissively synthesized *amH* virions display normal stage I and stage II DNA synthesis, but viral protein levels were dramatically reduced. Thus, to accurately determine whether a mutant H protein affects assembly, at least two H protein-mediated processes, DNA piloting and the stimulation of viral protein synthesis, cannot be affected or means to circumvent these functions must be employed.

During assembly, the internal scaffolding protein B facilitates the incorporation of one H protein into pentameric assembly intermediates (Chen et al., 2007; Cherwa et al., 2008; Novak and Fane, 2004). The association of 12 of these pentamers produces a $T=1$ virion that should have one H protein at each vertex; however, icosahedral averaging obscured the protein's direct visualization in atomic structures (Dokland et al., 1999; Dokland et al., 1997; McKenna et al., 1996; McKenna et al., 1994; McKenna et al., 1992). In the absence of a *bona fide* structure, bioinformatic analyses have provided some structural insights (Fig. 1). The protein contains a predicted N-terminal trans-membrane helix (Tusnady and Simon, 2001), which most likely functions during penetration, and several C-terminal coiled-coil domains (Lupas, 1996). Although coiled-coil domains are known to mediate protein oligomerization (Alfadhli et al., 2002; Harbury et al., 1993; Li et al., 2001), H protein oligomers have yet to be observed during the øX174 lifecycle.

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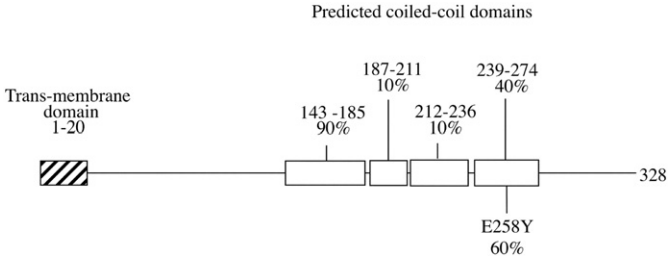

Fig. 1. Schematic of the øX174 H protein. The predicted trans-membrane and coiled-coil domains are depicted with hatched and white boxes, respectively, along with the amino acids predicted to be involved in secondary structure formation. The probability of each coiled-coil domain prediction (Lupas, 1996) for the wild-type protein is given atop the schematic, the probability prediction resulting from an E→Y substitution in the fourth domain is given below the schematic.

However, purified coiled-coil domains do form oligomers in solution (J. Nardozzi and G. Cingolani, personal communication). In a previous study, the coiled-coil domains were expressed in øX174 sensitive cells. Expression inhibited the piloting function of the wild-type H protein that enters the cell with the infecting genome (Ruboyianes et al., 2009). Overexpression of the internal scaffolding protein lessened the detrimental effects, which led to a hypothesis that protein B may moderate H protein oligomerization, perhaps keeping a subset of the protein in a monomeric state for assembly.

In this initial genetic analysis, preexisting *am(H)* strains were assayed for defective phenotypes in various informational suppressing (Su^+) hosts that insert non-wild-type amino acids during protein synthesis. The results of the analyses indicate that *am(H)* phenotypes in the SupD host are not a direct function of the synthesized missense proteins, as they are in SupE and SupF strains, but are highly influenced by host cell and phage physiology. This appears to be a unique characteristic of *am(H)* mutants, and was not observed with amber mutations in the other genes. One amber mutant produced a lethal phenotype in the SupF host. The corresponding missense mutation was placed directly into the genome and the resulting strain was characterized. The mutant protein removes assembly intermediates from the productive pathway: the first example of a mutant DNA pilot protein affecting morphogenesis. Overexpression of the internal scaffolding protein B gene restores productive assembly, which further emphasizes the role of B–H interactions during morphogenesis. However, the resulting lethal H protein-containing virions are significantly less infectious.

Results

The phenotype of am(H) mutants in SupD cells is primarily governed by general aspects of host cell and phage physiology, not the resulting missense protein

The plaque forming ability of *am(H)* mutant strains was examined on SupD, SupE and SupF hosts, which respectively insert serine, glutamine and tyrosine during protein synthesis. All of the *am(H)* mutants formed plaques on the SupE cell line (Table 1), and with the exception of *am(H)E258*, which is discussed below, formed plaques on the SupF host (data not shown). In contrast, all of the *am(H)* mutants appear to be restricted on the SupD host at 30 °C (Table 1: column $SupD^{ON}$), but will form plaques on this host at temperatures above 37 °C (data not shown). Bursts sizes (phage/cell produced in a single round of infection) were also measured at the restrictive 30 °C temperature as described in Materials and methods. Progeny production was assayed by titering on the permissive SupE host at 37 °C. As can be seen in Table 2, the *am(H)* mutants produced bursts in the SupD host at 30 °C. Moreover, a serine codon was placed directly in the *am(H)E258* genome at the site of the amber mutation. The phenotype of the resulting missense mutant was indistinguishable from wild-type in plating, attachment, assembly, and eclipse assays (data not shown).

Table 1
Plating efficiency[a] of øX174 amber mutants in SupD[b] and SupE cell lines at 30 °C.

Mutant[d]	Protein number[e]	Host cell[c] genotype genotype:			
		Su^-	SupE	$SupD^{ON}$	$SupD^{EXP}$
		(none)	(gln)	(ser)	(ser)
am(H)E258	12	10^{-6}	1.0	10^{-4}	0.5
am(H)Q26	12	10^{-6}	1.0	10^{-3}	1.0
am(H)Q89	12	$<10^{-3}$	1.0	10^{-3}	0.7
am(B)E60	60	$<10^{-3}$	1.0	0.9	0.8
am(D)Q11	240	$<10^{-3}$	0.8	1.0	0.8
am(F)S1	60	$<10^{-3}$	0.8	1.0	0.9
am(G)Q26	60	$<10^{-3}$	1.0	0.9	0.9
am(J)G3	60	$<10^{-3}$	0.8	1.0	0.6

[a] Plating efficiency is defined as assay titer/most permissive titer.
[b] The amino acids inserted by the informational suppressors are given in parentheses below the suppressors' names.
[c] ON and EXP indicate whether the cells in the plating assay were from an overnight (ON) or early exponential phase (EXP) culture.
[d] The letter in parentheses indicates the gene in which the amber mutation resides: H, DNA pilot protein; B, internal scaffolding protein; D, external scaffolding protein; F, coat protein; G, spike protein, J, DNA binding protein. The subsequent letter and number identify the wild-type amino codon and its position within the gene.
[e] Protein number indicates copy number of the protein in the virion and/or procapsid. Proteins F, G, and H are found in both virions and procapsids. Protein J is only present in virions. Scaffolding proteins B and D are found only in procapsids.

While burst experiments utilize cells in early exponential phase, standard plaque assays employ overnight cell cultures. Cells enter exponential phase after plates are incubated. To determine whether SupD cells must be in exponential phase to suppress *am(H)* mutants at lower temperatures, plating assays were repeated using early exponential phase SupD cells. As can be seen in Table 1 (column $SupD^{EXP}$), viability was restored. This phenomenon appears to be confined to SupD cells, the growth phase of SupE or SupF cells did not appear to affect *am(H)* plating efficiencies or plaque morphologies. Strains with amber mutations in the genes encoding the other virion and procapsid proteins were also assayed with stationary and early exponential phase SupD cells (Table 1). The growth phase of the plating cells affected neither plating efficiency nor plaque morphology. Thus, this phenomenon appears to be unique to *am(H)* mutants, even though it is the least abundant protein in virions and procapsids by a factor of 5 compared to the coat F, major spike G, internal scaffolding B and DNA binding J proteins or a factor of 20 compared to the external scaffolding D protein. This unique phenotype, which is exquisitely sensitive to host cell physiology, most likely reflects general aspects of øX174 biology (see Discussion).

The lethal phenotype of am(H)E258 in SupF cells is a function of the resulting missense protein

The *am(H)E258* mutation resides within the region of the gene encoding the fourth coiled-coil domain (Fig. 1). Theoretically, neither E→Q nor E→S substitutions; which would result from growth in SupE and SupD cells, respectively; alter the probability of coil formation (Lupas, 1996). However, the E→Y results in a much higher probability of coil

Table 2
Burst sizes (phage/cell) produced in single and co-infected cells at 30 °C.

Infection	Host cell genotype			
	SupE	SupD	SupF	Su^-
	(gln)	(ser)	(tyr)	(none)
am(H)E258	20	13	0.1	0.2
am(H)Q89	9.0	7.0	20	0.1
am(H)E258 and *am(H)Q89*	12	10	12	0.3
Wild-type[a]	43	71	44	76

[a] To ensure that all viral stocks were isogenic, the wild-type strain used in these studies was generated by recombination rescue as described in Materials and methods.