From Resistance to Stimulation: the Evolution of a Virus in the Presence of a Dominant Lethal Inhibitory Scaffolding Protein

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By acquiring resistance to an inhibitor, viruses can become dependent on that inhibitor for optimal fitness. However, inhibitors rarely, if ever, stimulate resistant strain fitness to values that equal or exceed the uninhibited wild-type level. This would require an adaptive mechanism that converts the inhibitor into a beneficial replication factor. Using a plasmid-encoded inhibitory external scaffolding protein that blocks \( \phi X174 \) assembly, we previously demonstrated that such mechanisms are possible. The resistant strain, referred to as the evolved strain, contains four mutations contributing to the resistance phenotype. Three mutations confer substitutions in the coat protein, whereas the fourth mutation alters the virus-encoded external scaffolding protein. To determine whether stimulation by the inhibitory protein coevolved with resistance or whether it was acquired after resistance was firmly established, the strain temporally preceding the previously characterized mutant, referred to as the intermediary strain, was isolated and characterized. The results of the analysis indicated that the mutation in the virus-encoded external scaffolding protein was primarily responsible for stimulating strain fitness. When the mutation was placed in a wild-type background, it did not confer resistance. The mutation was also placed in \( \psi \) with the plasmid-encoded dominant lethal mutation. In this configuration, the stimulating mutation exhibited no activity, regardless of the genotype (wild type, evolved, or intermediary) of the infecting virus. Thus, along with the coat protein mutations, stimulation required two external scaffolding protein genes: the once inhibitory gene and the mutant gene acquired during evolution.

With developed genetics and biochemistry and solved atomic structures, bacteriophage \( \phi X174 \) has a long and rich history as a virus assembly system (15–17, 20, 22–25). Due to its rapid replication, which allows selective pressures to be applied for hundreds of infection cycles, it recently emerged as an attractive organism for evolutionary studies (4–6, 33–35). An evolutionary approach to assembly was used to study the evolution of resistance to a genetically engineered inhibitory protein, a dominant lethal external scaffolding protein that specifically targets procapsid morphogenesis. A multiple-mutant resistant strain was experimentally evolved by culturing \( \phi X174 \) in exponential-phase cells while incrementally increasing the induction of the lethal dominant gene (12). Like other viruses that acquire resistance to antiviral agents (14, 27, 28, 37), the resistant strain exhibits lower fitness than that of the uninhibited wild-type strain in the absence of the inhibitor. It is also dependent on the once inhibitory protein for optimal fitness, which is not uncommon (3, 29). But unlike similar examples, the inhibitor stimulates fitness to values equal to or above the uninhibited wild-type level, suggesting that the virus evolved a mechanism to convert the inhibitory protein into a beneficial replication factor.

The inhibitory external scaffolding protein was designed to inhibit conformational switching during assembly. In the procapsid atomic structure (15, 16), there are four structurally unique external scaffolding subunits (D1 to D4) associated with each viral coat protein (Fig. 1). The subunits are arranged as dimers of asymmetric dimers (\( D_1D_2-D_3D_4 \)). To achieve this unique arrangement, which is unrelated to quasi-equivalence, one monomer of each asymmetric dimer, \( D_1 \) and \( D_3 \), must be bent 30° in \( \alpha \)-helix 3. This vital kink, which is also present in the assembly of the naïve D protein dimer crystal structure (25), is mediated by glycine residue 61 (G61). The torsional angles necessary for kinking require a glycine residue, as all other amino acid side chains occupy forbidden regions within the Ramachandran plot. The expression of cloned D genes containing various G61 substitutions inhibits wild-type morphology. The severity of inhibition roughly correlates with the size of the substituted side chain (13). Alone, the mutant proteins appear to have no activity vis-à-vis the ability to interact with other viral proteins. Wild-type D subunits are required to bring them into the assembly pathway, suggesting that the inhibitory species is a heterodimer that siphons early assembly intermediates into an insoluble fraction.

Although strains resistant to the lethal proteins were isolated easily in one-step genetic selections, the resistance phenotype was weak (13), as determined by plating assays. To isolate a strain with a robust resistance phenotype, \( \phi X174 \) was cultured continually on exponential-phase cells while the induction of the lethal dominant gene was increased incrementally (12). The experimentally evolved strain has five mutations: three confer substitutions in the coat protein (D34H, D205N, and S227P), one confers a substitution in the DNA pilot protein (D136G), and one confers a substitution in the external scaffolding protein (D34G). The coat protein mutations are the primary determinants of resistance, whereas the altered DNA pilot protein most likely acts on the level of higher eclipse rates (12). The results of the genetic analyses...
presented here demonstrate that the scaffolding protein mutation may not contribute directly to the resistance phenotype but may act as a compensatory mutation elevating strain fitness.

As described above, the strain is dependent upon this once inhibitory protein, which stimulates resistant strain fitness to levels equaling or exceeding the uninhibited wild-type level. Here we address whether stimulation coevolved with resistance or whether it was acquired in one distinct genetic step after resistance was firmly established. To answer this question, the strain that temporally preceded the previously characterized resistance strain was isolated and characterized. The results of the analysis indicated that the mutation in the genomic copy of the external scaffolding gene was primarily responsible for stimulating strain fitness. Subsequently, this mutation did not confer resistance in an otherwise wild-type background, nor did it display activity when placed in cis with the dominant lethal mutation at the G61 site. Moreover, the system may demonstrate how mutations in a duplicated gene product can lead to morphogenetic complexity, in this case, to a three-scaffolding-protein system, with one internal scaffolding protein and two versions of the external scaffolding protein.

MATERIALS AND METHODS

Phage plating, media, buffers, stock preparation, and fitness assays. The reagents, media, buffers, and protocols for plating and stock preparation (18) and fitness assays (6) have been described previously. All fitness assays were conducted concurrently in triplicate and used the same sources of all viruses and cells. Data were analyzed to determine statistical significance by use of GraphPad Prism software.

Bacterial strains, plasmids, and phage strains. The Escherichia coli C strains C122 and BAF30 (recA) have been described previously (18, 19).

The generation of the multiple mutant resistant to the expression of the G61D mutant external scaffolding protein was described in a previous study (12). It was generated by serial passage of dX174 through exponential-phase cells while incrementally increasing the expression of the lethal dominant G61D gene. The strain that preceded the evolved strain, referred to as the intermediary strain, was isolated by picking single plaques from earlier cultures, which were preserved at −80°C in 15% glycerol. Entire genome sequences were determined and compared to that of the most evolved strain. Fragments of approximately 1,000 nucleotides were amplified by PCR and sequenced from both ends by the DNA sequencing core at the University of Arizona. Sequencing was performed on both populations and purified plaques. See Results and Discussion for details. The intermediary/nullID strain, which contains an amber mutation in codon 22 of the D gene, was generated by oligonucleotide-mediated mutagenesis as previously described (21). A strain containing only the D34G mutation, the mh3d(D)/D34G strain, was generated by recombination rescue of a nullID strain (8) with a cloned D gene containing the D34G mutation (see below). The utilization strains, i.e., the ut3h/Fs/h426L/nullID and ut3h(B)/y1099/nullID strains, which are capable of producing infectious virions when complemented by the G61A mutant external scaffolding protein, have been described previously (11), as has the isolation of the mh3d(B)/P102L strain (13). The mh3d(B)/P102L(P)/R233H mutant was isolated by plating the mh3d(B)/P102L strain on cells expressing the D34G/G61D mutant gene.

The construction of the cloned wild-type D gene (pND) and the inhibitory D genes (pG61D, pG61K, and pG61A) has been described previously (11, 13). To construct cloned D genes containing the D34G mutation with and without mutations in the G61 codon, the 5′ end of the D gene was amplified using the DNA of the evolved strain as a template. The upstream primer introduced a BglII site. The downstream primers introduced a SacII site and mutations in the D gene expression produces protein levels that approximate 25% of the total intracellular D protein (12).

RESULTS AND DISCUSSION

Stimulation by the once inhibitory G61D mutant external scaffolding protein did not coevolve with resistance but was achieved in one genetic step. To determine if the observed stimulation in fitness coevolved with the resistance phenotype or was acquired by a single genetic event, the strain that temporally preceded the most evolved strain during experimental evolution was identified and isolated as described in Materials and Methods. The complete genome sequence of this strain, referred to as the intermediary strain, was determined and compared to that of the “fully” evolved strain from the previous study. The only difference between the two strains was the D34G mutation, which confers a D→G substitution in α-helix 2 of the external scaffolding D protein. This mutation was found only in the most evolved strain. Thus, it was the last to arise during the prolonged selection process.

Although resistance to an inhibitor and stimulation by it are most likely intertwined phenomena, the D34G mutation may be involved primarily in the latter. Unlike the coat protein mutations, the D34G mutation was not recovered as a resistance mutation in exhaustive one-step genetic selections (13). To assay the effects of the D34G mutation in stimulating fitness, fitness assays were conducted with the ancestor, intermediary, and evolved strains, using previously defined conditions that result in maximal fitness, which occurs when cloned D gene expression produces protein levels that approximate 25% of the total intracellular D protein (12).

All fitness data were generated simultaneously in triplicate, using the same sources of cells and viruses. Thus, all values can be compared directly. Data are presented in two forms: fitness values (number of doublings/h) and numbers of progeny produced (number of progeny/h/input phage).

In the absence of exogenous D gene expression, both the evolved and intermediary strains demonstrated a decrease in fitness compared to the wild-type ancestor. The fitness differences between the intermediary and evolved strains were not statistically significant, indicating that the three coat protein mutations were largely responsible for this reduction (Table 1, no exogenous D protein expression, and Fig. 2). As expected, the expression of the inhibitory G61D D protein was detrimental to the wild-type ancestor strain, resulting in negative fitness
The inability of strain; EV, most evolved strain; M, 

The corresponding yields of progeny, expressed as numbers of virions/h/input virus, are given in Table 1. For graphing

The stimulating D34G mutation does not confer resistance in an otherwise wild-type background. The D34G mutation was also placed in a wild-type background, and the resulting strain, the mh3d(D)D34G strain, was assayed for the resistance phenotype. As can be seen in Table 1, this mutation alone did not confer resistance to the G61D mutant, as determined by fitness assays. In plaque assays, which are much more sensitive at detecting low levels of viability, the mh3d(D)D34G mutant exhibited a plating efficiency of $10^{-6}$, a value identical to that exhibited by the wild-type ancestor. The inability of the D34G mutation alone to confer even the slightest degree of resistance to the G61D protein suggests that its selective advantage is dependent on the presence of the coat protein mutations.

High levels of stimulation require the combination of the D34G and G61D mutant proteins. To determine whether stimulation was observed with another D protein containing a lethal dominant substitution for G61, evolved strain fitness was measured in cells expressing another G61 allele, G61K, and the wild-type protein (Table 1 and Fig. 2). The expression of the wild-type D gene had a positive effect on fitness for the ancestor, evolved, and intermediary strains, resulting in approximately 3- to 4-fold elevated virion yields (Table 1, exogenous wild-type D gene expression, and Fig. 2). This result was expected, as preinduction of the cloned wild-type D gene is known to increase yields by reducing the lag phase before virion production in wild-type infections (30–32). However, this stimulation was far less dramatic than the 100-fold stimulation observed for the evolved strain in cells expressing the G61D mutant external scaffolding protein gene. Thus, a high level of stimulation appeared to be a property of combining the G61D and D34G mutant proteins.

Stimulation requires the D34G and G61D mutations to be present in trans. Experiments were performed to determine
the configuration and combinations of the various mutations that would stimulate strain fitness to the uninhibited wild-type level. The fitness of the ancestor, intermediary, and evolved strains was measured in cells producing the D34G/G61D protein, in which the two mutations are present in cis, and the D34G protein. As can be seen in Table 1 and Fig. 2, only the evolved strain exhibited an elevation in fitness in cells expressing the D34G and D34G/G61D genes. However, this positive effect on strain fitness was considerably lower than that observed with expression of the G61D gene. The expression of the D34G gene was inhibitory to the ancestor, whereas its effects on the intermediary strain were minimal. These data suggest that the combination of the wild-type and D34G effects on the intermediary strain were minor. The presence of the D34G mutation in cis with mutations at G61 may result in a less functional or more inhibitory D protein. To investigate this further, the behavior of a single-mutant resistant strain was examined. The mh3d(B)P102L mutant confers the strongest resistance phenotype against the G61D protein among previously isolated single mutants (13), but it was not resistant to the expression of the D34G/G61A and D34G/G61D genes. However, resistance was achieved by the acquisition of a second mutation in the coat protein, an R→H substitution at position 233 [mh3d(B)P102L/(F)R233H strain].

TABLE 2. Plating efficiencies of nullD and intermediary/nullD strains, previously isolated G61A utilizer strains, and previously isolated G61D resistant strains in cells expressing exogenous D genes with the D34G mutation in cis with G61D and G61A mutations

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plating efficiency (assay titer/permisive titer)* with indicated exogenous D gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>nullD</td>
<td>&lt;10^{-4}</td>
</tr>
<tr>
<td>intermediary/nullD</td>
<td>&lt;10^{-3}</td>
</tr>
<tr>
<td>ut3d(F)S426L/nullD</td>
<td>&lt;10^{-4}</td>
</tr>
<tr>
<td>ut3d(B)H109Y/nullD</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>mh3d(B)P102L</td>
<td>1.0</td>
</tr>
<tr>
<td>mh3d(B)P102L/(F)R233H</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Permissive titers were determined in cells expressing the wild-type D gene.

- The D34G and G61D mutations were present in cis for the experiments described in the preceding paragraph. However, the D34G/G61D protein was not the only external scaffolding protein in the infected cells. Either a wild-type or a D34G protein was also present. To categorically determine if the D34G mutation in cis with mutations at G61 may result in a less functional or more inhibitory D protein. To investigate this further, the behavior of a single-mutant resistant strain was examined. The mh3d(B)P102L mutant confers the strongest resistance phenotype against the G61D protein among previously isolated single mutants (13), but it was not resistant to the expression of the D34G/G61A and D34G/G61D genes. However, resistance was achieved by the acquisition of a second mutation in the coat protein, an R→H substitution at position 233 [mh3d(B)P102L/(F)R233H strain].

**Evolution of multiple scaffolding protein systems.** While most large double-stranded DNA bacteriophages and many eukaryotic viruses rely upon a single internal scaffolding protein, the small single-stranded DNA microviruses are unique, as they require two scaffolding proteins for assembly, namely, an internal and an external species. While both proteins are essential in a wild-type background, the results of genetic and structural studies indicate that the external scaffolding protein is more critical for morphogenesis (7, 9, 15, 16, 26). Indeed, targeted genetic selections have been used to isolate a φX174 sextuple mutant that no longer requires the internal scaffolding protein for viability (10). Thus, a two-scaffolding-protein system can be evolved in a stepwise fashion into a one-scaffolding-protein system. Since the optimal fitness of the evolved strain appears to be dependent upon two distinct species of mutant external scaffolding proteins, this may represent the evolution of a three-scaffolding-protein system. If this was the case, then optimal fitness would require (i) some allele specificity vis-à-vis the two utilized external scaffolding proteins and (ii) the D34G and G61D mutations to be present in trans, on separate proteins, not in cis. The results indicate that these criteria have been met.

The inhibitory G61D protein, which is known to promiscuously promote off-pathway reactions (11, 13), decreases the lag phase before virions are produced in cells infected with the most evolved strain (12). The coat protein resistance mutations may produce early assembly intermediates that are less prone to associate with scaffolding subunits, both wild type and inhibitory. Thus, efficient nucleation may now depend on a small concentration of the more reactive inhibitory proteins. Unlike infections with the most evolved strain, overall yields of the intermediary strain are not increased. This suggests that the
D34G mutation alleviates defects in capsid elongation efficiency or fidelity. In the nonquasi-equivalent external scaffolding lattice found in the procapsid (Fig. 1), the residues directly adjacent to G61 in α-helix 3 mediate D1-D2 and D3-D4 intradimer contacts and interdimer contacts between the D1 and D3 subunits (15, 16). Moreover, α-helix 3 residues in subunits D2, D3, and D4 mediate D-D contacts across the 2-fold axes of symmetry. Residue 34 is located in α-helix 2. This helix is in contact with α-helix 3 in every subunit. Since mutations at D34 and G61 must be in trans to stimulate fitness, the D34G mutation may provide added flexibility to subunits adjacent to those with a G61 mutation. Although the structural and genetic complexity of the system confines insights to the speculative level, the virus has evolved a means to productively incorporate the once inhibitory protein into its life cycle, and the mechanism requires the interaction of two distinct mutant external scaffolding proteins.

Gene duplication has long been identified as a major mechanism that drives evolution (36) and is often used to explain the genetic composition of extant viruses. For example, the three coat proteins and genes of picornaviruses are believed to have a common origin (2). In the Microviridae, the major spike protein may have arisen from a duplication of the coat protein gene (24). However, in these examples, extensive genetic modifications have occurred since the hypothesized duplication event, whereas in the system described herein, the duplication event was recent and subsequent adaptations could be documented specifically and temporally. While the behavior of the evolved strain appears to meet the criteria of a three-scaffolding-protein system, one that could arise soon after the duplication of a gene product, one of the external scaffolding proteins is encoded on a plasmid. For a bona fide system to evolve, gene D would need to be duplicated within the genome. This would require the genome to be increased by approximately 8.5%, which would lead to the production of considerably fewer stable particles (1). Thus, additional mutations to stabilize the capsid would be required. Clearly, the feasibility of this evolutionary path is difficult to predict. However, since one of the external scaffolding genes is expressed as a component of the host cell’s genetic material, albeit from a plasmid, the system can be regarded within the context of host-specific adaptations and/or the coopting of a host cell protein for the parasite’s advantage.

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REFERENCES


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