The Loop Formed by Residues 340 to 343 of Reovirus μ 1 Controls Entry-Related Conformational Changes

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ABSTRACT Reovirus particles are covered with 200 μ 1/ σ 3 heterohexamers. Following attachment to cell surface receptors, reovirus is internalized by receptor-mediated endocytosis. Within the endosome, particles undergo a series of stepwise disassembly events. First, the σ 3 protector protein is degraded by cellular proteases to generate infectious subviral particles (ISVPs). Second, the μ 1 protein rearranges into a protease-sensitive conformation to generate ISVP*s and releases two virus-encoded peptides, μ 1N and Φ . The released peptides promote delivery of the genome-containing core by perforating the endosomal membrane. Thus, to establish a productive infection, virions must be stable in the environment but flexible to disassemble in response to the appropriate cellular cue. The reovirus outer capsid is stabilized by μ 1 intratrimer, intertrimer, and trimer-core interactions. As a consequence of ISVP-to-ISVP* conversion, neighboring μ 1 trimers unwind and separate. Located within the μ 1 jelly roll β barrel domain, which is a known regulator of ISVP* formation, residues 340 to 343 form a loop and have been proposed to facilitate viral entry. To test this idea, we generated recombinant reoviruses that encoded deletions within this loop (Δ 341 and Δ 342). Both deletions destabilized the outer capsid. Notably, Δ 342 impaired the viral life cycle; however, replicative fitness was restored by an additional change (V403A) within the μ 1 jelly roll β barrel domain. In the Δ 341 and Δ 342 backgrounds, V403A also rescued defects in ISVP-to-ISVP* conversion. Together, these findings reveal a new region that regulates reovirus disassembly and how perturbing a metastable capsid can compromise replicative fitness.

IMPORTANCE Capsids of nonenveloped viruses are composed of protein complexes that encapsulate, or form a shell around, nucleic acid. The protein-protein interactions that form this shell must be stable to protect the viral genome but also sufficiently flexible to disassemble during cell entry. Thus, capsids adopt conformations that undergo rapid disassembly in response to a specific cellular cue. In this work, we identify a new region within the mammalian orthoreovirus outer capsid that regulates particle stability. Amino acid deletions that destabilize this region impair the viral replication cycle. Nonetheless, replicative fitness is restored by a compensatory mutation that restores particle stability. Together, this work demonstrates the critical balance between assembling virions that are stable and maintaining conformational flexibility. Any factor that perturbs this balance has the potential to block a productive infection.

KEYWORDS capsid, conformational change, metastable, nonenveloped virus, reovirus, thermostability

Capsids of nonenveloped and enveloped viruses serve at least two antagonistic functions. First, the protein-protein, protein-nucleic acid, and/or protein-lipid interactions that stabilize the capsid must be sufficiently strong to allow particle assembly and stability within the environment. This protection function is essential for preserving and transmitting the genomic material (1). Second, in response to one or more cues

Received 31 May 2017 Accepted 2 August 2017

Accepted manuscript posted online 9 August 2017

Citation Snyder AJ, Danthi P. 2017. The loop formed by residues 340 to 343 of reovirus μ 1 controls entry-related conformational changes. *J Virol* 91:e00898-17. <https://doi.org/10.1128/JVI.00898-17>.

Editor Susana López, Instituto de Biotecnología/UNAM

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from the cell (e.g., pH or protein-protein and/or protein-lipid interactions), the capsid must disassemble and deliver its genome to the appropriate cellular compartment (1). If either of the functions is out of balance, the virus will be unable to initiate a productive infection and/or spread to a new host. Mammalian orthoreovirus (reovirus) is a versatile experimental model system for virus entry studies (2). Like other viruses, reovirus has evolved to maintain the proper balance between capsid stability and flexibility.

Reovirus particles are composed of two concentric protein shells: the inner capsid (core), which encapsidates 10 segments of genomic, double-stranded RNA, and the outer capsid, which contains all of the proteins necessary for host cell attachment and entry (2–4). The outer capsid consists of 200 $\mu 1/\sigma 3$ heterohexamers, which are arranged in an incomplete T=13 lattice. The $\sigma 3$ protector protein caps $\mu 1$, presumably to prevent premature disassembly. Each $\mu 1$ monomer is intertwined with two others to form a trimer. Within fully assembled particles, $\mu 1$ is composed of a jelly roll β barrel domain that connects to a core-adjacent, α -helical pedestal (3–5). As demonstrated by heat-, ethanol-, and phenol-resistant strains (6–12), the outer capsid is stabilized by $\mu 1$ -mediated intratrimer, intertrimer, and trimer-core interactions. Thus, to allow efficient particle assembly and environmental stability, contacts between neighboring $\mu 1$ monomers must maintain structural integrity; however, the lattice must also undergo efficient disassembly during cell entry.

Reovirus entry is characterized by a series of stepwise disassembly events. Following attachment to proteinaceous or carbohydrate receptors, such as junctional adhesion molecule A, Ngr1, or glycans (13–19), virions are internalized by receptor-mediated endocytosis (20–24). Within the endosome, acid-dependent cathepsin proteases degrade the $\sigma 3$ outer capsid protein (24–30). This step is accompanied by exposure and cleavage of the cell penetration protein, $\mu 1$, into $\mu 1\delta$ and the C-terminal Φ fragment (31). The cleaved, metastable intermediate is called an infectious subviral particle (ISVP) (3). Within certain organs, such as the gastrointestinal tract, ISVPs are generated extracellularly by chymotrypsin (32–34). Reovirus undergoes a second structural transition to deliver the genome-containing core into the host cytoplasm. The conformationally altered particle is called ISVP* (35). This transition is characterized by unwinding and separation of neighboring $\mu 1$ trimers (5, 36) and by autocatalytic cleavage of the $\mu 1$ N-terminal fragment, $\mu 1N$, from $\mu 1\delta$ (4, 37, 38). In the ISVP configuration, $\mu 1N$ and Φ are located proximal to the core (3–5); however, ISVP-to-ISVP* conversion liberates these fragments from the virion (4, 35, 37–42). The released fragments are thought to facilitate core delivery by generating size-selective pores within the host endosomal membrane (39, 41, 43). Although the in-cell trigger of ISVP-to-ISVP* conversion is not fully established, this transition is induced *in vitro* using nonspecific factors, such as heat and large monovalent cations (9, 35, 44, 45) or factors that ISVPs are more likely to encounter during entry, such as released $\mu 1N$ and lipids (46–48).

In this work, we took a genetic and biochemical approach to investigate the balance between the stabilizing and genome delivery functions of reovirus capsids. Located within the $\mu 1$ jelly roll β barrel domain, residues 340 to 343 form a loop and have been proposed to facilitate viral entry (5). To test this idea, we generated single-residue deletions (termed $\Delta 341$ and $\Delta 342$) within the T1L/T3D M2 genetic background. T1L (reovirus type 1 Lang) and T3D (reovirus type 3 Dearing) represent the prototype strains for their respective mammalian orthoreovirus serotypes; T1L $\times$ T3D reassortant viruses are used extensively to investigate many aspects of the reovirus replication cycle (2). As such, we used T1L/T3D M2, which contains a T3D-derived M2 gene (encoding $\mu 1$) and nine genes from T1L, as the parental virus (i.e., wild type). We show the following: (i) in the absence or presence of lipid membranes, deleting residue 341 or 342 from the $\mu 1$ jelly roll β barrel domain destabilized the reovirus outer capsid; (ii) enhanced capacity to undergo ISVP-to-ISVP* conversion correlated with impaired replication and/or spread; and (iii) a second-site change (V403A) within $\mu 1$, in combination with the original residue 342 deletion, rescued replicative fitness. Together, this work further underscores the role of the $\mu 1$ jelly roll β barrel in regulating capsid stability.

FIG 1 Sequences, protein compositions, and size distributions of the μ 1 340–343 deletion mutants. (A) μ 1 amino acid sequence alignments. Residues corresponding to the 340–343 loop are in boldface. (B) Protein compositions. Virions and chymotrypsin-generated ISVPs of T1L/T3D M2, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 342 were analyzed by SDS-PAGE. The gel was Coomassie brilliant blue stained. Migration of reovirus capsid proteins is indicated on the left of the gel. μ 1 resolves as μ 1C, and μ 1 δ resolves as δ (38). μ 1N and Φ are too small to resolve on the gel. (C) Particle size distribution profiles. Virions and chymotrypsin-generated ISVPs of T1L/T3D M2, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 342 were analyzed by dynamic light scattering. For each virus, the virion and ISVP size distribution curves are overlaid ($n = 3$ independent replicates for each virus; the results of one representative experiment are shown).

RESULTS

Recombinant reoviruses with deletions in the μ 1 340–343 loop. The μ 1 jelly roll β barrel domain regulates the stability of the reovirus outer capsid (6–12). Residues 340 to 343 within this domain form a loop and have been proposed to facilitate cell entry by interacting with negatively charged lipid head groups (see Fig. 6A to C) (5). We recently demonstrated that certain lipid formulations promote entry-related conformational changes (47, 48); however, whether these changes occur via an interaction with the μ 1 340-to-343 (340–343) loop is unclear. Thus, we sought to test the idea that residues 340 to 343 regulate ISVP-to-ISVP* conversion through an interaction with lipids. Toward this end, we generated recombinant reoviruses that contain deletions in the μ 1 340–343 loop, termed Δ 341 and Δ 342 (Fig. 1A). All the viruses were made in the T1L/T3D M2 background, which contains a wild-type or mutated T3D M2 gene (encoding μ 1) and nine genes from T1L. We generated viruses in this genetic background to build upon our previous studies on the regulation of cell entry events using variants of T1L/T3D M2 (9, 10, 49–51). Compared to the wild type, T1L/T3D M2 Δ 341 and T1L/T3D M2 Δ 342 displayed no observable defects in protein composition or stoichiometry (Fig. 1B). To rule out the possibility of gross structural changes, we analyzed virions and ISVPs by dynamic light scattering (DLS) (Fig. 1C). For each virus, we detected a single peak at the expected hydrodynamic diameter (data not shown).

Deletions within the μ 1 340–343 loop destabilize the reovirus outer capsid. During host entry, reovirus ISVPs must undergo a conformational change to deposit the genome-containing core into the host cytoplasm. The conformationally altered particle is called ISVP* (4, 35, 37–42). *In vitro*, ISVP-to-ISVP* conversion can be induced using nonphysiological physical or chemical triggers, such as heat and large monovalent cations (9, 35, 44, 45). Inducing this structural transition with heat renders the particles noninfectious (45). To test if the μ 1 340–343 loop regulates ISVP-to-ISVP* conversion, we conducted 5-min heat inactivation experiments over a range of temperatures (Fig. 2A). Following incubation at 39°C, T1L/T3D M2 Δ 341 ISVPs and T1L/T3D M2 Δ 342 ISVPs were reduced in titer by $\sim 2.0 \log_{10}$ units and $\sim 3.0 \log_{10}$ units, respectively, relative to control virus that was incubated at 4°C. In contrast, T1L/T3D M2 ISVPs were reduced in titer by $\sim 2.0 \log_{10}$ units after incubation at 42°C. Concurrent with ISVP-to-ISVP* conversion, the reovirus outer capsid protein μ 1 undergoes a conformational change that renders μ 1 susceptible to proteolysis (35). This structural rearrangement is assayed *in vitro* by heating ISVPs and determining the susceptibility of the δ fragment (a product

FIG 2 Deletions within the μ 1 340–343 loop are thermolabilizing. (A and C) Thermal inactivation. T1L/T3D M2, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 342 ISVPs at 2×10^{12} particles/ml were incubated in virus storage buffer in the absence (A) or presence (C) of EE liposomes for 5 min at the indicated temperatures. The change in infectivity relative to samples incubated at 4°C was determined by plaque assay. The data are presented as means $\pm$ SD. *, $P \leq 0.05$ ($n = 3$ independent replicates for each reaction condition). (B and D) Heat-induced ISVP-to-ISVP* conversion. T1L/T3D M2, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 342 ISVPs at 2×10^{12} particles/ml were incubated in virus storage buffer in the absence (B) or presence (D) of EE liposomes for 5 min at the indicated temperatures. Each reaction mixture was then treated with trypsin for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were analyzed by SDS-PAGE. The gels were Coomassie brilliant blue stained ($n = 3$ independent replicates for each reaction condition; the results of one representative experiment are shown).

of μ 1 cleavage) to trypsin digestion (35, 45). The μ 1 340–343 deletion mutants were incubated under conditions identical to those in Fig. 2A. Consistent with the thermal-inactivation results, deleting residue 341 or 342 from μ 1 was thermolabilizing (Fig. 2B). The δ fragment in T1L/T3D M2 Δ 341 ISVPs and in T1L/T3D M2 Δ 342 ISVPs became trypsin sensitive at 39°C, whereas the δ fragment in T1L/T3D M2 ISVPs became trypsin sensitive at 42°C.

The data presented above indicate that deleting residue 341 or 342 from μ 1 destabilized ISVPs and enhanced ISVP-to-ISVP* conversion. Lipids also lower the temperature needed to induce this conformational change for two prototype reovirus strains (e.g., T1L and T3D) (47, 48). Δ 341 and Δ 342 are predicted to collapse the μ 1

340–343 loop, rendering ISVPs less susceptible to lipid-driven ISVP* formation. To evaluate the effects of lipids on the mutant reovirus strains, we conducted heat inactivation experiments in the presence of early endosome (EE) liposomes (Fig. 2C); EE liposomes resemble the lipid composition of early endosomal membranes (47, 52). Following incubation at 36°C, T1L/T3D M2 Δ 341 ISVPs and T1L/T3D M2 Δ 342 ISVPs were reduced in titer by $\sim 3.5 \log_{10}$ units and $\sim 5.5 \log_{10}$ units, respectively. In contrast, T1L/T3D M2 ISVPs were reduced in titer by $\sim 5.0 \log_{10}$ units after incubation at 39°C. The thermal-inactivation results were confirmed using the trypsin sensitivity assay (Fig. 2D). Contrary to expectations, T1L/T3D M2 Δ 341 and T1L/T3D M2 Δ 342 remained sensitive to lipids (i.e., lipid-driven ISVP* formation occurred at a lower temperature than in the wild type). Together, these data suggest that either the predicted function of the μ 1 340–343 loop is incorrect or deleting residue 341 or 342 is insufficient to impair the proposed lipid-interacting function. Nonetheless, the characterization of T1L/T3D M2 Δ 341 and T1L/T3D M2 Δ 342 revealed a previously undescribed region of μ 1 that regulates ISVP-to-ISVP* conversion.

As a consequence of ISVP-to-ISVP* conversion, reovirus particles release μ 1 N- and C-terminal fragments, μ 1N and Φ , respectively (4, 35, 37–42). The released peptides generate pores within endosomal membranes, which are thought to mediate core delivery to the host cytoplasm (39, 41, 43). Thus, membrane penetration can be used as an indirect measure for ISVP* formation. *In vitro*, ISVPs can induce hemolysis of (i.e., generate pores within) bovine red blood cells (RBCs). The extent of RBC hemolysis correlates with the extent of ISVP-to-ISVP* conversion (9, 35). To test if deletions within the μ 1 340–343 loop affect ISVP-induced hemolysis, T1L/T3D M2 Δ 341 ISVPs or T1L/T3D M2 Δ 342 ISVPs were incubated with RBCs for 1 h at 37°C (Fig. 3A). Comparing wild-type virus to each mutant, we observed similar levels of hemolysis. Nonetheless, when we performed the trypsin sensitivity assay, the δ fragment in T1L/T3D M2 Δ 341 ISVPs and T1L/T3D M2 Δ 342 ISVPs became trypsin sensitive faster (2.5 min postincubation) than the δ fragment in T1L/T3D M2 ISVPs (5 min postincubation) (Fig. 3B). These results suggest that small differences in ISVP-to-ISVP* conversion may not allow measurable differences in hemolysis. Thus, we reduced the efficiency of ISVP* formation by incubating the reaction mixtures for 1 h at 30°C (Fig. 3C). Under these conditions, T1L/T3D M2 Δ 342 ISVPs induced significantly more hemolysis than T1L/T3D M2 ISVPs or T1L/T3D M2 Δ 341 ISVPs. Moreover, the δ fragment in T1L/T3D M2 Δ 342 ISVPs became trypsin sensitive (i.e., adopted the ISVP* conformation) 20 min faster than the δ fragment in wild-type virus (Fig. 3D).

Deletions within the μ 1 340–343 loop do not affect the ISVP*-promoting activity of released μ 1N. ISVP* formation culminates in the release of two μ 1-derived fragments, μ 1N and Φ , and the cell attachment protein, σ 1 (4, 35, 37–42). In addition to generating pores within target membranes (39, 41, 43), μ 1N, and to a lesser extent Φ , facilitates ISVP-to-ISVP* conversion *in trans* (46, 48). The μ 1 340–343 deletion mutants are more thermolabile than wild-type virus (Fig. 2 and 3). To determine whether the released fragments from T1L/T3D M2 Δ 341 and T1L/T3D M2 Δ 342 promote thermal inactivation more efficiently than the released fragments from wild-type virus, T1L/T3D M2 ISVPs were incubated with the supernatant of preconverted ISVP*s. ISVP* supernatant, which contains μ 1N, Φ , and σ 1 (41), was generated from T1L/T3D M2, T1L/T3D M2 Δ 341, or T1L/T3D M2 Δ 342 by incubating ISVPs for 5 min at 52°C (41, 46). This temperature was selected to ensure that each strain underwent ISVP* formation (i.e., each strain released an equivalent amount of the released fragments). The reactions were centrifuged to pellet particles, and the supernatant (spin) was transferred to tubes containing T1L/T3D M2 ISVPs. The supernatant was analyzed by plaque assay (Fig. 4A) and by Western blotting (Fig. 4B) to confirm the clearance of input ISVPs. T1L/T3D M2 ISVPs were incubated with the indicated ISVP* supernatant for 5 min over a range of temperatures (Fig. 4C). As expected, ISVPs mixed with ISVP* supernatant inactivated at a lower temperature than ISVPs alone. Moreover, the released components from T1L/T3D M2, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 342 equally reduced the titer of input ISVPs (e.g., $\sim 2.0 \log_{10}$ units at 36°C). These results were confirmed using

FIG 3 Deletions within the $\mu 1$ 340–343 loop enhance ISVP-induced hemolysis. (A and C) ISVP-induced hemolysis. T1L/T3D M2, T1L/T3D M2 $\Delta 341$, or T1L/T3D M2 $\Delta 342$ ISVPs at 2×10^{12} particles/ml were incubated with bovine RBCs in virus storage buffer for 1 h at 37°C (A) or 30°C (C). After 1 h, hemolysis was quantified by measuring the absorbance of the supernatant at 405 nm. Levels of 0 and 100% hemolysis were determined by incubating an equivalent number of RBCs in virus storage buffer or virus storage buffer supplemented with RBCs and 0.8% Triton X-100, respectively. The data are presented as means and SD, $^* P \leq 0.05$ ($n = 3$ independent replicates for each reaction condition). (B and D) RBC-induced ISVP-to-ISVP* conversion. T1L/T3D M2, T1L/T3D M2 $\Delta 341$, or T1L/T3D M2 $\Delta 342$ ISVPs at 2×10^{12} particles/ml were incubated with RBCs in virus storage buffer for the indicated amounts of time at 37°C (B) or 30°C (D). The supernatant of each reaction mixture was then treated with trypsin for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were analyzed for the presence of the $\mu 1$ δ fragment by Western blotting ($n = 3$ independent replicates for each reaction condition; the results of one representative experiment are shown).

the trypsin sensitivity assay (Fig. 4D). When ISVP* supernatant was included in the reaction, the δ fragment in the input ISVPs became trypsin sensitive at 33°C. Together, these results suggest that deletions within the $\mu 1$ 340–343 loop destabilize the reovirus outer capsid rather than directly enhancing the ISVP*-promoting activity of released $\mu 1$ N.

Reovirus with deletion of residue 342 in the $\mu 1$ 340–343 loop displays small-plaque morphology. The reovirus outer capsid contains 200 $\mu 1$ trimers, which are arranged in an incomplete T=13 lattice (3–5). This lattice, which is surface exposed in ISVPs, is stabilized by intra- and intertrimer interactions (6–11). We hypothesized that the strengths of these interactions are inversely proportional to the capacity to undergo ISVP-to-ISVP* conversion (10). Deletions within the $\mu 1$ 340–343 loop destabilize the outer capsid (Fig. 2 and 3); however, neither T1L/T3D M2 $\Delta 341$ nor T1L/T3D M2 $\Delta 342$ displayed significant defects in the particle/PFU ratio or in the onset (or levels) of viral protein synthesis following infection (data not shown). Nonetheless, plaque assays containing chymotrypsin in the overlay indicated that deletion of residue 342 impaired one or more steps of the viral life cycle (e.g., entry, replication, spread, and/or host response to virus infection) (Fig. 5). Compared to T1L/T3D M2, T1L/T3D M2 $\Delta 342$ produced a greater proportion of small plaques. In contrast, T1L/T3D M2 $\Delta 341$, which was more thermostable than T1L/T3D M2 $\Delta 342$ (Fig. 2 and 3), displayed only a modest decrease in average plaque size (Fig. 5).

FIG 4 The $\mu 1$ 340–343 deletion mutants retain ISVP*-promoting activity. (A and B) Generation of ISVP* supernatant. Input T1L/T3D M2, T1L/T3D M2 $\Delta 341$, or T1L/T3D M2 $\Delta 342$ ISVPs at 2×10^{12} particles/ml were incubated for 5 min at 52°C. The heat-inactivated virus (no spin) was centrifuged to pellet particles. The supernatant (spin) was immediately transferred to tubes containing target T1L/T3D M2 ISVPs for thermal-inactivation reactions. Aliquots of the no-spin and spin reaction mixtures were analyzed for residual infectivity by plaque assay (A) and for the presence of the $\mu 1$ δ fragment by Western blotting (B). In panel A, the data are presented as means and SD; *, $P \leq 0.05$ ($n = 3$ independent preparations of ISVP* supernatant analyzed). (C) ISVP* supernatant-mediated thermal inactivation. T1L/T3D M2 ISVPs at 2×10^{12} particles/ml were incubated in virus storage buffer supplemented with the indicated ISVP* supernatants for 5 min at the indicated temperatures. The change in infectivity relative to samples incubated at 4°C was determined by plaque assay. The data are presented as means $\pm$ SD; *, $P \leq 0.05$ ($n = 3$ independent replicates for each reaction condition). (D) ISVP* supernatant-mediated ISVP-to-ISVP* conversion. T1L/T3D M2 ISVPs at 2×10^{12} particles/ml were incubated in virus storage buffer supplemented with the indicated ISVP* supernatants for 5 min at the indicated temperatures. Each reaction mixture was then treated with trypsin for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were analyzed by SDS-PAGE. The gels were Coomassie brilliant blue stained ($n = 3$ independent replicates for each reaction condition; the results of one representative experiment are shown).

Maintaining the balance between capsid stability and conformational flexibility is an essential component of the viral life cycle. To identify residues that regulate this balance, T1L/T3D M2 $\Delta 342$ was serially passaged, and revertants were selected based on the capacity to generate large plaques compared to the parental virus (Fig. 5). As such, only large-plaque variants were isolated for amplification and sequencing. Because T1L/T3D M2 $\Delta 341$ produced plaques that were medium to large, the virus was not used for revertant screening. Most thermostabilizing and thermolabilizing mutations are found in $\mu 1$ (6–11). Thus, we sequenced the entire $\mu 1$ -encoding M2 gene. Among six large plaques sequenced, half reverted to wild type. The remaining plaques contained the original mutation (i.e., $\Delta 342$) and an additional, second-site change, V403A. Based on the crystal structure of the T1L $\mu 1$ homotrimer (5) (Fig. 6A to C), V403A and residues 340 to 343 are not expected to physically interact or mediate intra- or intertrimer interactions. Nonetheless, both are located within the $\mu 1$ jelly roll β barrel domain, which is known to regulate ISVP-to-ISVP* conversion (6–12).

The V403A second-site change restores wild-type-like plaque morphology. To determine whether V403A alone was responsible for the revertant (i.e., large-plaque) phenotype, we reintroduced the second-site change into T1L/T3D M2 $\Delta 342$. This step

FIG 5 Plaque morphologies of the $\mu 1$ 340–343 loop deletion mutants. Purified T1L/T3D M2 $\Delta 342$ was serially passaged in L cells, and the presence of second-site reversions was monitored by plaque size. Plaque assays were fixed with formaldehyde, stained with crystal violet, and washed with water at 5 days postinfection. Plaques of purified T1L/T3D M2, T1L/T3D M2 $\Delta 341$, and T1L/T3D M2 $\Delta 342$ (left) and twice-passaged T1L/T3D M2 $\Delta 342$ (right) are shown.

was needed to ensure that additional genetic alterations were not present in the remaining gene segments. V403A was also introduced into T1L/T3D M2 and T1L/T3D M2 $\Delta 341$. Compared to the wild type, these viruses displayed no observable defects in protein composition or stoichiometry (Fig. 6D). To rule out the possibility of gross structural changes, we analyzed virions and ISVPs by dynamic light scattering (Fig. 6E). For each virus, we detected a single peak at the expected hydrodynamic diameter (data not shown). We next compared the plaque sizes of T1L/T3D M2 V403A, T1L/T3D M2 $\Delta 341$ V403A, and T1L/T3D M2 $\Delta 342$ V403A to that of the wild type (Fig. 6F). The plaque morphology of T1L/T3D M2 V403A resembled that of T1L/T3D M2, whereas T1L/T3D M2 $\Delta 341$ V403A and T1L/T3D M2 $\Delta 342$ V403A produced mostly large plaques. These results indicate the following: (i) V403A alone does not overtly impact the viral life cycle (compare plaque sizes for T1L/T3D M2 and T1L/T3D M2 V403A) and (ii) V403A rescues the defect that causes small-plaque morphology for T1L/T3D M2 $\Delta 342$ (compare Fig. 5 and 6F).

The V403A second-site change stabilizes the reovirus outer capsid. Our initial characterization of the $\mu 1$ 340–343 mutants indicated that these viruses were more thermolabile than the wild type (Fig. 2 and 3). To determine if the second-site change rescued this phenotype, we incubated three different viruses that encode V403A (Fig. 6) for 5 min over a range of temperatures (Fig. 7A). Following incubation at 45°C, T1L/T3D M2 ISVPs and T1L/T3D M2 V403A ISVPs were reduced in titer by $\sim 3.5 \log_{10}$ units. In contrast, T1L/T3D M2 $\Delta 341$ V403A ISVPs and T1L/T3D M2 $\Delta 342$ V403A ISVPs were reduced in titer by $\sim 2.5 \log_{10}$ units and $\sim 1.0 \log_{10}$ unit, respectively. Thus, V403A allows enhanced resistance to thermal inactivation (i.e., ISVP-to-ISVP* conversion) (compare Fig. 2 and 7). These results were confirmed using the trypsin sensitivity assay (Fig. 7B). The δ fragment in T1L/T3D M2 ISVPs and T1L/T3D M2 V403A ISVPs became trypsin sensitive at 42°C, whereas the δ fragment in T1L/T3D M2 $\Delta 341$ V403A ISVPs and T1L/T3D M2 $\Delta 342$ V403A ISVPs became trypsin sensitive at 45°C and 48°C, respectively.

As discussed above, lipids promote ISVP-to-ISVP* conversion in a lipid composition-dependent manner (47, 48). To determine if V403A is thermostabilizing when lipids are present, we conducted heat inactivation experiments in the presence of EE liposomes (Fig. 7C). Following incubation at 42°C, T1L/T3D M2 ISVPs and T1L/T3D M2 V403A ISVPs were reduced in titer by $\sim 5.0 \log_{10}$ units. In contrast, T1L/T3D M2 $\Delta 341$ V403A ISVPs and T1L/T3D M2 $\Delta 342$ V403A ISVPs were reduced in titer by $\sim 2.0 \log_{10}$ units and $\sim 1.0 \log_{10}$ unit, respectively. Furthermore, the δ fragment in T1L/T3D M2 ISVPs and T1L/T3D M2 V403A ISVPs became trypsin sensitive at 39°C, whereas the δ fragment in T1L/T3D M2 $\Delta 341$ V403A ISVPs and T1L/T3D M2 $\Delta 342$ V403A ISVPs became trypsin sensitive at 42°C and 45°C, respectively (Fig. 7D).

We next sought to determine if V403A reduces the capacity of ISVPs to induce hemolysis. Viruses that encode the second-site change (Fig. 6) were incubated with RBCs for 1 h at 37°C (Fig. 8A). Compared to T1L/T3D M2 ISVPs, we observed a significant decrease in hemolysis for T1L/T3D M2 $\Delta 341$ V403A ISVPs and T1L/T3D M2 $\Delta 342$ V403A

FIG 6 Protein compositions, size distributions, and plaque sizes of the μ 1 V403A mutants. (A) Side and top views (left and right, respectively) of the T1L μ 1 homotrimer (5) (Protein Data Bank [PDB] accession number 1JMU). Individual μ 1 monomers are colored blue, red, and green. Residues corresponding to the μ 1 340–343 loop are represented by gray spheres. μ 1 residue V403 is represented by black spheres. (B and C) Structure of the T1L μ 1 monomer (5) (PDB accession number 1JMU). Purple, μ 1N; teal, Φ ; orange, δ . Residues corresponding to the μ 1 340–343 loop are represented by gray spheres. μ 1 residue V403 is represented by black spheres. (C) Enlarged views of the T1L μ 1 jelly roll β barrel domain. (D) Protein compositions. Virions and chymotrypsin-generated ISVPs of T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A were analyzed by SDS-PAGE. The gel was Coomassie brilliant blue stained. Migration of reovirus capsid proteins is indicated on the left of the gel. μ 1 resolves as μ 1C, and μ 1 δ resolves as δ (38). μ 1N and Φ are too small to resolve on the gel. (E) Particle size distribution profiles. Virions and chymotrypsin-generated ISVPs of T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A were analyzed by dynamic light scattering. For each virus, the virion and ISVP size distribution curves are overlaid ($n = 3$ independent replicates for each virus; the results of one representative experiment are shown). (F) Plaque sizes. L cell monolayers were infected with T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, or T1L/T3D M2 Δ 342 V403A ISVPs. At 5 days postinfection, plaque assays were fixed with formaldehyde, stained with crystal violet, and washed with water.

ISVPs. Moreover, the δ fragment in wild-type virus became trypsin sensitive 7.5 min faster than the δ fragment in viruses that contained V403A and deletions within the μ 1 340–343 loop (Fig. 8B). Together, these results highlight the role of the μ 1 jelly roll β barrel domain in regulating ISVP-to-ISVP* conversion (i.e., capsid stability) and subsequent pore formation.

Deleting residue 342 from the μ 1 340–343 loop impairs replicative fitness.

Defects in ISVP-to-ISVP* conversion (Fig. 2 and 3) correlate with reduced plaque size (Fig. 5). To determine if deletion of residue 342 affects replication efficiency, we generated single- and multistep growth profiles. Murine L929 (L) cells were infected at high or low multiplicity of infection (MOI), and the viral yield was quantified at the indicated times postinfection. Following infection at high MOI (10 PFU/cell) (Fig. 9A), T1L/T3D M2 and T1L/T3D M2 Δ 342 produced similar growth profiles. We next infected cells at low MOI (0.01 PFU/cell) (Fig. 9B). Under these conditions, multiple rounds of

FIG 7 The μ 1 V403A second-site change is thermostabilizing. (A and C) Thermal inactivation. T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A ISVPs at 2×10^{12} particles/ml were incubated in virus storage buffer in the absence (A) or presence (C) of EE liposomes for 5 min at the indicated temperatures. The change in infectivity relative to samples incubated at 4°C was determined by plaque assay. The data are presented as means $\pm$ SD, *, $P \leq 0.05$ ($n = 3$ independent replicates for each reaction condition). (B and D) Heat-induced ISVP-to-ISVP* conversion. T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A ISVPs at 2×10^{12} particles/ml were incubated in virus storage buffer in the absence (B) or presence (D) of EE liposomes for 5 min at the indicated temperatures. Each reaction mixture was then treated with trypsin for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were analyzed by SDS-PAGE. The gels were Coomassie brilliant blue stained ($n = 3$ independent replicates for each reaction condition; the results of one representative experiment are shown).

infection were expected to occur. We did not observe a significant difference in growth between T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 341 V403A (data not shown). In contrast, T1L/T3D M2 Δ 342 produced fewer infectious units than the wild type by 48 and 72 h postinfection. Nonetheless, replicative fitness was

FIG 8 The μ 1 V403A second-site change inhibits ISVP-induced hemolysis. (A) ISVP-induced hemolysis. T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A ISVPs at 2×10^{12} particles/ml were incubated with bovine RBCs in virus storage buffer for 1 h at 37°C. After 1 h, hemolysis was quantified by measuring the absorbance of the supernatant at 405 nm. Levels of 0 and 100% hemolysis were determined by incubating equivalent numbers of RBCs in virus storage buffer or virus storage buffer supplemented with RBCs and 0.8% Triton X-100, respectively. The data are presented as means and SD; *, $P \leq 0.05$ ($n = 3$ independent replicates for each reaction condition). (B) RBC-induced ISVP-to-ISVP* conversion. T1L/T3D M2, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A ISVPs at 2×10^{12} particles/ml were incubated with RBCs in virus storage buffer for the indicated amounts of time at 37°C. The supernatant of each reaction mixture was then treated with trypsin for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were analyzed for the presence of the μ 1 δ fragment by Western blotting ($n = 3$ independent replicates for each reaction condition; the results of one representative experiment are shown).

restored by the V403A second-site change (Fig. 9B, compare T1L/T3D M2 and T1L/T3D M2 Δ 342 V403A). These data indicate that altered ISVP-to-ISVP* conversion correlates with reduced plaque size and reduced virus growth.

DISCUSSION

Reovirus entry is characterized by a series of stepwise disassembly events: (i) virions are converted to ISVPs by cellular proteases (24–30) and (ii) ISVPs undergo a significant conformational change to generate ISVP*s (4, 35, 37–42). In this work, we explored the role of the reovirus μ 1 340–343 loop in regulating ISVP-to-ISVP* conversion. Viruses that encoded deletions within this loop (Fig. 1), Δ 341 and Δ 342, were more unstable (i.e., converted to ISVP*s at a lower temperature) than the wild type (Fig. 2) and had enhanced hemolytic activity (Fig. 3). Thus, disrupting the μ 1 340–343 loop destabilized the outer capsid. Any factor that alters capsid stability is expected to negatively impact replicative fitness (6, 8, 10); capsids must be stable to protect the viral genome but also

FIG 9 Growth profiles of the μ 1 340–343 loop deletion mutants. L cell monolayers were infected with T1L/T3D M2, T1L/T3D M2 Δ 342, or T1L/T3D M2 Δ 342 V403A virions at an MOI of 10 PFU/cell (A) or 0.01 PFU/cell (B). At the indicated times postinfection, the infected cells were lysed and the viral yield was quantified by plaque assay. The data are presented as means and SD; *, $P \leq 0.05$ ($n = 3$ independent replicates for each infection condition).

flexible to undergo rapid disassembly during cell entry (1). Compared to the wild type, which produced small, medium, and large plaques, $\Delta 342$ produced only small and medium plaques (Fig. 5). Moreover, $\Delta 342$ produced reduced viral yields following multiple rounds of infection (Fig. 9). Nonetheless, replicative fitness was restored by a second-site change (V403A) within the $\mu 1$ jelly roll β barrel domain (Fig. 6 and 9). In the $\Delta 341$ and $\Delta 342$ backgrounds, V403A also rescued defects in ISVP-to-ISVP* conversion (Fig. 7) and inhibited ISVP-induced hemolysis (Fig. 8). Together, these results further underscore the critical balance between capsid stability and conformational flexibility.

Heat induces multiple structural transitions within ISVPs to generate ISVP*s (9, 35, 45). Notably, two pore-forming peptides, $\mu 1N$ and Φ , are released from the virus particle (4, 35, 37–42). These peptides are thought to facilitate core delivery by generating size-selective pores within the endosomal membrane (39, 41, 43). Following attachment to host cell receptors (13–18), reovirus is internalized by receptor-mediated endocytosis (20–24). Only particles that traffic through the early and late endosomes are capable of establishing a productive infection (53). Thus, premature conversion to ISVP*s is expected to render the particle noninfectious. Based on reduced plaque size (Fig. 5) and reduced virus growth (Fig. 9), deleting residue 342 from the $\mu 1$ 340–343 loop impairs one or more steps of the viral life cycle. Moreover, in the absence or presence of lipids, the $\Delta 342$ mutant is significantly more thermolabile than the wild type (Fig. 2). There are at least two explanations for the observed defect in replicative fitness. First, the $\Delta 342$ mutant undergoes premature ISVP-to-ISVP* conversion following internalization. As a consequence, released $\mu 1N$ and Φ would be unable to localize to the appropriate cellular membrane. Second, the $\Delta 342$ mutant undergoes ISVP-to-ISVP* conversion prior to host cell attachment. The plaque assays used in this work incorporated chymotrypsin into the overlay; ISVPs are generated within the medium and must remain stable until the virus attaches to and infects a new cell. Similar conditions occur within the gastrointestinal tract, where ISVPs are generated extracellularly by chymotrypsin (32–34). Thus, due to its thermolabile nature (Fig. 2 and 3), a significant percentage of newly released $\Delta 342$ particles may be rendered noninfectious (i.e., undergo ISVP-to-ISVP* conversion) prior to attachment. Nonetheless, the $\Delta 342$ mutant did not show a significant defect in the particle/PFU ratio or in the onset (or levels) of viral protein synthesis following infection (data not shown), suggesting small defects in ISVP* formation and/or spread may only be evident by plaque morphology (Fig. 5) and by multicycle growth analysis (Fig. 9).

The reovirus outer capsid is stabilized by $\mu 1$ -mediated intratrimer, intertrimer, and trimer-core interactions (6–12). Studies to isolate heat-resistant strains identified two types of thermostabilizing mutations: (i) changes within or near intratrimer or intertrimer contacts and (ii) changes on the outer surface (i.e., solvent exposed) of $\mu 1$ (6, 8, 10). In each case, enhanced thermostability was correlated with increased resistance to ISVP-to-ISVP* conversion. Moreover, reoviruses that encoded K459E, K407R, or D371A (all mutations in $\mu 1$) were more thermostable than the wild type and displayed defects in particle infectivity, such as reduced plaque size (6, 8). Remarkably, the majority of thermolabilizing pseudoreversions, which reversed heat-resistant phenotypes, were also near the trimer-trimer interface (6). The $\mu 1$ 340–343 loop and V403A are not expected to directly interact, nor are these residues expected to mediate intersubunit contacts. Nonetheless, each region is solvent exposed within the $\mu 1$ jelly roll β barrel domain (Fig. 6). Located on the outermost surface (i.e., opposite the core) of the reovirus outer capsid, this domain regulates membrane penetration by controlling the efficiency of ISVP-to-ISVP* conversion (6–12). Although the precise mechanism by which the $\mu 1$ 340–343 loop and V403A influence capsid stability is unclear, the upper portions of neighboring $\mu 1$ trimers are thought to unwind and separate during the onset of ISVP* formation (5, 36). Presumably, $\Delta 341$ and $\Delta 342$ deletions destabilize the $\mu 1$ jelly roll β barrel domain, allowing these conformational changes to occur more readily. V403 has not been previously identified as a site that controls heat resistance. Unlike D371A, which rescues numerous thermolabilizing mutations (6), V403A could be uniquely positioned to compensate for the $\mu 1$ 340–343 loop.

Within the Δ 341 or Δ 342 background, V403A rescued defects in ISVP-to-ISVP* conversion (Fig. 7) and inhibited hemolytic activity (Fig. 8). Moreover, virus with both mutations (e.g., Δ 342 and V403A) underwent entry-related conformational changes at a higher temperature than T1L/T3D M2. Despite their hyperstable nature, these viruses produced plaques (Fig. 6) and displayed growth profiles (Fig. 9) that resembled those of the wild type. As stated above, capsids must be stable to protect the viral genome but also flexible to undergo rapid disassembly; however, the proper balance is likely dependent upon environmental conditions. At higher temperatures or when ISVPs are generated extracellularly, hyperstable variants of μ 1 (i.e., those that display reduced ISVP-to-ISVP* conversion efficiency) are likely more favored than flexible variants. We made a similar observation using thermolabilizing mutations within the μ 1 α -helical pedestal (10). Virus with the original mutation (e.g., E89A or E89Q) and a thermostabilizing second-site change converted to ISVP*s at a higher temperature than the wild type. Additional studies (*in vitro* and *in vivo*) are needed to fully characterize the effects of hyperstable variants on replicative fitness.

In addition to being required for membrane penetration, μ 1 regulates the induction of cell death (54–58); however, reoviruses that encode apoptosis-defective variants of μ 1 maintain replicative fitness (54, 55). Thus, it seems unlikely that reduced virus growth (Fig. 9) is related to the cell death-inducing properties of μ 1. As a major reovirus structural protein, μ 1 also influences particle assembly (2); it could be argued that deletion of residue 342 confers two defects, (i) an unstable capsid that permits facile ISVP-to-ISVP* conversion (Fig. 2 and 3) and (ii) improper assembly. The V403A second-site change restored the wild-type-like plaque phenotype (Fig. 6), capsid stability (Fig. 7 and 8), and replicative fitness (Fig. 9). Given the low likelihood that V403A rescues multiple steps of the viral replication cycle, these data suggest a model in which the defect is due to altered ISVP-to-ISVP* conversion.

Studies thus far have identified numerous thermostabilizing and thermolabilizing mutations within the reovirus μ 1 protein (6, 8, 10). In many cases, such as the work presented here, perturbing the balance between a stable-flexible capsid impairs one or more steps of the viral life cycle. *In vitro*, these mutations modulate the efficiency at which the outer capsid undergoes entry-related conformational changes. Although a subset of residues regulate intratrimer and intertrimer interactions, certain regions may also influence the interaction between ISVPs and released μ 1N. In addition to generating pores within target membranes (39, 41, 43), μ 1N can promote ISVP-to-ISVP* conversion *in trans* (46, 48). Thus, future studies are needed to identify the μ 1N binding site on target ISVPs and to assess if this mechanism is relevant to a bona fide infection.

MATERIALS AND METHODS

Cells and viruses. Murine L929 (L) cells were grown at 37°C in Joklik's minimal essential medium (Lonza, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA), and 25 ng/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). All virus strains used in this study were derived from reovirus type 3 Dearing (T3D) and reovirus type 1 Lang (T1L) and were generated by plasmid-based reverse genetics (59, 60). Mutations within the T3D M2 gene were generated by QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). Primer sequences are available upon request.

Virus purification. Recombinant reovirus strains T1L/T3D M2, T1L/T3D M2 Δ 341, T1L/T3D M2 Δ 342, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, and T1L/T3D M2 Δ 342 V403A, which all contain a wild-type or mutated T3D M2 gene in an otherwise T1L background, were propagated and purified as previously described (9, 61). Briefly, L cells infected with second-passage reovirus stocks were lysed by sonication. Virus particles were extracted from the lysates using Vertrel-XF specialty fluid (Dupont, Wilmington, DE) (62). The extracted particles were layered onto 1.2- to 1.4-g/cm³ CsCl step gradients. The gradients were then centrifuged at $187,000 \times g$ for 4 h at 4°C. Bands corresponding to purified virus particles (~ 1.36 g/cm³) (63) were isolated and dialyzed into virus storage buffer (10 mM Tris, pH 7.4, 15 mM MgCl₂, and 150 mM NaCl). Following dialysis, the particle concentration was determined by measuring the optical density of the purified virus stocks at 260 nm (OD₂₆₀) (1 unit at OD₂₆₀ is equal to 2.1×10^{12} particles/ml) (63).

Generation of ISVPs. T1L/T3D M2, T1L/T3D M2 Δ 341, T1L/T3D M2 Δ 342, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, or T1L/T3D M2 Δ 342 V403A virions (2×10^{12} particles/ml or 4×10^{12}

particles/ml) were digested with 200 $\mu\text{g/ml}$ TLCK (*N* α -*p*-tosyl-L-lysine chloromethyl ketone)-treated chymotrypsin (Worthington Biochemical, Lakewood, NJ) in a total volume of 100 μl for 20 min at 32°C (64, 65). After 20 min, the reaction mixtures were incubated for 20 min on ice and quenched by the addition of 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). The generation of ISVPs was confirmed by SDS-PAGE and Coomassie brilliant blue staining.

DLS. T1L/T3D M2, T1L/T3D M2 Δ 341, T1L/T3D M2 Δ 342, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, or T1L/T3D M2 Δ 342 V403A virions or ISVPs (2×10^{12} particles/ml) were analyzed using a Zetasizer Nano S dynamic-light-scattering system (Malvern Instruments, Malvern, United Kingdom). All measurements were made at room temperature in a quartz Suprasil cuvette with a 3.00-mm path length (Hellma Analytics, Mullheim, Germany). For each sample, the hydrodynamic diameter was determined by averaging readings across 15 iterations.

Liposome preparation. The lipids used in this study (L- α -phosphatidylcholine [PC] from chicken egg, sphingomyelin [SM] from porcine brain, cholesterol [Chl] from ovine wool, L- α -phosphatidylethanolamine [PE] from chicken egg, L- α -phosphatidylserine [PS] from porcine brain, and lysobisphosphatidic acid [LBPA]) were purchased from Avanti Polar Lipids (Alabaster, AL). All the lipids were dissolved in chloroform and stored at -20°C . Prior to liposome preparation, the lipids were dried under a stream of argon gas; 1 mM liposomes were prepared by resuspending the dried lipids in 250 μl of virus storage buffer (10 mM Tris, pH 7.4, 15 mM MgCl_2 , and 150 mM NaCl) and passing the resuspension (31 times) through an Avanti Mini Extruder with a 0.1- μm -pore-size polycarbonate membrane (Avanti Polar Lipids, Alabaster, AL). The composition of EE liposomes was Chl-PC-PE-SM-PS-LBPA (50:26:13:5:5:1 molar ratio) (47, 48, 52).

Thermal-inactivation and trypsin sensitivity assays. T1L/T3D M2, T1L/T3D M2 Δ 341, T1L/T3D M2 Δ 342, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, or T1L/T3D M2 Δ 342 V403A ISVPs (2×10^{12} particles/ml) were incubated in the absence or presence of 1 mM EE liposomes for 5 min at the indicated temperatures in a Bio-Rad S1000 (Hercules, CA) thermal cycler. When specified, ISVP* supernatant (15 μl of supernatant per 30 μl of total reaction volume) (Fig. 4) was included. The total volume of each reaction mixture was 30 μl in virus storage buffer (10 mM Tris, pH 7.4, 15 mM MgCl_2 , and 150 mM NaCl). For each reaction condition, an aliquot was also incubated for 5 min at 4°C . Following incubation, 10 μl of each reaction mixture was diluted in 40 μl of ice-cold virus storage buffer (10 mM Tris, pH 7.4, 15 mM MgCl_2 , and 150 mM NaCl), and infectivity was determined by plaque assay. The change in infectivity at a given temperature (T) was calculated using the following formula: $\log_{10}(\text{PFU/ml})_T - \log_{10}(\text{PFU/ml})_{4^\circ\text{C}}$. Under each reaction condition, the titers of the 4°C control samples were between 5×10^9 and 5×10^{10} PFU/ml. The remaining 20 μl of each reaction mixture was treated with 0.08 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO) for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were solubilized in reducing SDS sample buffer and analyzed by SDS-PAGE. The gels were Coomassie brilliant blue stained (Sigma-Aldrich, St. Louis, MO) and imaged on an Odyssey imaging system (Li-Cor, Lincoln, NE).

Plaque assay. Plaque assays to determine infectivity were performed as previously described (45, 61). Briefly, control or heat-treated virus samples or infected cell lysates were diluted in phosphate-buffered saline (PBS) supplemented with 2 mM MgCl_2 . L cell monolayers in 6-well plates (Greiner Bio-One, Kremsmünster, Austria) were infected with 250 μl of diluted virus or infected cell lysate for 1 h at room temperature. Following the viral-attachment incubation, the monolayers were overlaid with 4 ml of serum-free medium 199 (Sigma-Aldrich, St. Louis, MO) supplemented with 1% Bacto agar (BD Biosciences, Franklin Lakes, NJ), 10 $\mu\text{g/ml}$ TLCK-treated chymotrypsin (Worthington Biochemical, Lakewood, NJ), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 $\mu\text{g/ml}$ streptomycin (Invitrogen, Carlsbad, CA), and 25 ng/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). The infected cells were incubated at 37°C , and plaques were counted 5 days postinfection. When indicated, plaque assays were fixed with 3.7% formaldehyde and stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO) dissolved in 5% ethanol. The fixed and stained cells were then washed with water.

ISVP-induced hemolysis assay. Citrated bovine RBCs (Colorado Serum Company, Denver, CO) were pelleted for 5 min by centrifugation at $500 \times g$ and resuspended in ice-cold PBS supplemented with 2 mM MgCl_2 (PBS^{Mg}). This step was repeated until the supernatant remained clear after pelleting. After washing, the RBCs were resuspended in PBS^{Mg} at a 30% (vol/vol) concentration. Hemolysis efficiency was determined by incubating a suboptimal number of T1L/T3D M2, T1L/T3D M2 Δ 341, T1L/T3D M2 Δ 342, T1L/T3D M2 V403A, T1L/T3D M2 Δ 341 V403A, or T1L/T3D M2 Δ 342 V403A ISVPs (2×10^{12} particles/ml) in a 3% (vol/vol) solution of RBCs. The levels of 0 and 100% hemolysis were determined by incubating an equivalent number of RBCs in virus storage buffer (10 mM Tris, pH 7.4, 15 mM MgCl_2 , and 150 mM NaCl) or in virus storage buffer supplemented with 0.8% Triton X-100, respectively. The samples were incubated for 1 h at the indicated temperatures (Fig. 3A and C and 8A) or for the indicated amounts of time at the indicated temperatures (Fig. 3B and D and 8B). Following incubation, the reaction mixtures were placed on ice for 20 min, followed by centrifugation at $500 \times g$ for 5 min at 4°C . To quantify the amount of hemoglobin release (Fig. 3A and C and 8A), the supernatants were diluted 1:5 in virus storage buffer (10 mM Tris, pH 7.4, 15 mM MgCl_2 , and 150 mM NaCl), and the absorbance at 405 nm (A_{405}) was measured using a microplate reader (Molecular Devices, Sunnyvale, CA). Percent hemolysis was calculated using the following formula: $[(A_{\text{sample}} - A_{\text{buffer}})/(A_{\text{TX-100}} - A_{\text{buffer}})] \times 100$, where A_{sample} is the absorbance of the sample, A_{buffer} is the absorbance of the buffer, and $A_{\text{TX-100}}$ is the absorbance of the buffer supplemented with RBCs and 0.8% Triton X-100.

When indicated (Fig. 3B and D and 8B), the supernatant from hemolysis reactions (20 μ l) was treated with 0.08 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO) for 30 min on ice. Following digestion, equal numbers of particles from each reaction mixture were solubilized in reducing SDS sample buffer. The samples were analyzed for the presence of the μ 1 δ fragment by Western blotting using a rabbit anti-reovirus (α -reovirus) polyclonal antibody (66).

Generation of ISVP* supernatant. The supernatant of preconverted ISVP*s was generated as previously described (41, 46). Briefly, input T1L/T3D M2, T1L/T3D M2 Δ 341, or T1L/T3D M2 Δ 342 ISVPs (2×10^{12} particles/ml) were incubated for 5 min at 52°C. The heat-inactivated virus (no spin) was then centrifuged at $16,000 \times g$ for 10 min at 4°C to pellet particles. The supernatant (spin) was immediately transferred to tubes containing target T1L/T3D M2 ISVPs for thermal-inactivation reactions. To ensure that intact ISVPs did not contaminate the transferred ISVP* supernatant, aliquots of the no-spin and spin reaction mixtures were analyzed for residual infectivity by plaque assay and for the presence of the μ 1 δ fragment by Western blotting using a rabbit α -reovirus polyclonal antibody (66).

Revertant screening. Purified T1L/T3D M2 Δ 342 was serially passaged in L cells, and the presence of second-site reversions was monitored by plaque size. For each passage, 50 μ l of inoculum from the previous passage was diluted in 300 μ l of PBS supplemented with 2 mM $MgCl_2$. The diluted inoculum was used to infect L cells in 60-mm dishes (TPP, Trasadingen, Switzerland). The infected cells were incubated at 37°C until all the cells detached. To generate lysates, infected cells were frozen at $-80^\circ C$ and then thawed at room temperature twice. The viral lysates were analyzed by plaque assay.

Revertants with large-plaque phenotypes were selected by plaque purification and amplified in L cells to obtain viral stocks. Viral RNA from the selected revertants was isolated by lysing infected cells with TRI Reagent (Molecular Research Center, Cincinnati, OH), followed by phenol-chloroform extraction. The extracted RNA was subjected to reverse transcription (RT)-PCR using M2 gene segment-specific primers. The PCR products were resolved on Tris-acetate-EDTA agarose gels, purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany), and sequenced using primers that covered the entire length of the M2 open reading frame. Primer sequences are available upon request. To verify that the large-plaque phenotype was due to the mutation identified in sequencing, the revertant site was introduced back into T1L/T3D M2, T1L/T3D M2 Δ 341, and T1L/T3D M2 Δ 342. These viruses were purified as described above and analyzed for defects in plaque size, thermal inactivation, ISVP-to-ISVP* conversion, hemolysis, and virus growth.

Single- and multicycle growth assays. L cell monolayers in 6-well plates (Greiner Bio-One, Kremsmünster, Austria) were infected with T1L/T3D M2, T1L/T3D M2 Δ 342, or T1L/T3D M2 Δ 342 V403A virions (10 PFU/cell [Fig. 9A] or 0.01 PFU/cell [Fig. 9B]) for 1 h at room temperature. Following the viral-attachment incubation, the monolayers were washed three times with PBS and overlaid with 2 ml of Joklik's minimal essential medium (Lonza, Walkersville, MD) supplemented with 5% FBS (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA), and 25 ng/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). The infected cells were either lysed immediately by two freeze-thaw cycles (0-h time point) or incubated at 37°C. At the indicated times postinfection, the incubated cells were lysed and the virus titer was determined by plaque assay. The viral yield at a given time postinfection (t) was calculated using the following formula: $\log_{10}(\text{PFU/ml})_t - \log_{10}(\text{PFU/ml})_{0 \text{ h}}$. Following infection with 10 PFU/cell, the titers of the 0-h control samples were between 1×10^5 and 4×10^5 PFU/ml. Following infection with 0.01 PFU/cell, the titers of the 0-h control samples were between 1×10^2 and 3×10^2 PFU/ml.

Statistical analyses. The reported values represent the means of three independent biological replicates. The error bars indicate standard deviations (SD). P values were calculated using Student's t test (two-tailed; unequal variance assumed).

Modeling. Molecular graphics were created and analyses were performed with the UCSF Chimera package (67).

ACKNOWLEDGMENTS

We thank the members of our laboratory and the Indiana University Bloomington virology community for helpful suggestions and reviews of the manuscript. Dynamic light scattering was performed in the Indiana University Bloomington Physical Biochemistry Instrumentation Facility.

The research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award numbers 1R01AI110637 (to P.D.) and F32AI126643 (to A.J.S.) and by Indiana University Bloomington. The content is solely our responsibility and does not necessarily represent the official views of the funders.

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