Reovirus Variants Selected for Resistance to Ammonium Chloride Have Mutations in Viral Outer-Capsid Protein $\sigma 3$

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Mammalian reoviruses are internalized into cells by receptor-mediated endocytosis. Within the endocytic compartment, the viral outer capsid undergoes acid-dependent proteolysis resulting in removal of the $\sigma 3$ protein and proteolytic cleavage of the $\mu 1/\mu 1C$ protein. Ammonium chloride (AC) is a weak base that blocks disassembly of reovirus virions by inhibiting acidification of intracellular vacuoles. To identify domains in reovirus proteins that influence pH-sensitive steps in viral disassembly, we adapted strain type 3 Dearing (T3D) to growth in murine L929 cells treated with AC. In comparison to wild-type (wt) T3D, AC-adapted (ACA-D) variant viruses exhibited increased yields in AC-treated cells. AC resistance of reassortant viruses generated from a cross of wt type 1 Lang and ACA-D variant ACA-D1 segregated with the $\sigma 3$ -encoding S4 gene. The deduced $\sigma 3$ amino acid sequences of six independently derived ACA-D variants contain one or two mutations each, affecting a total of six residues. Four of these mutations, I180T, A246G, I347S, and Y354H, cluster in the virion-distal lobe of $\sigma 3$. Linkage of these mutations to AC resistance was confirmed in experiments using reovirus disassembly intermediates recoated with wt or mutant $\sigma 3$ proteins. In comparison to wt virions, ACA-D viruses displayed enhanced susceptibility to proteolysis by endocytic protease cathepsin L. Image reconstructions of cryoelectron micrographs of three ACA-D viruses that each contain a single mutation in the virion-distal lobe of $\sigma 3$ demonstrated native capsid protein organization and minimal alterations in $\sigma 3$ structure. These results suggest that mutations in $\sigma 3$ that confer resistance to inhibitors of vacuolar acidification identify a specific domain that regulates proteolytic disassembly.

Receptor-mediated endocytosis plays an essential role in the internalization of many ligands and is involved in receptor-linked signaling pathways, recycling of cell membranes, and antigen presentation (41). Many viruses require endocytic uptake and exposure to acidic pH or acid-dependent proteases to productively infect host cells. For enveloped viruses like dengue virus (32, 51), influenza virus (9, 11, 55), and Sindbis virus (26, 53), acid-dependent conformational changes involving envelope glycoproteins are required for membrane fusion. Endocytic uptake and acidification are also required for entry of some nonenveloped viruses such as adenovirus (31, 60), astrovirus (20), parvovirus (7), and reovirus (40, 56). In addition, proteolysis of certain capsid components by viral or host pro-

teases is required for entry of some of these viruses. The precise mechanisms by which endosomal acidification and proteolysis of viral capsid components mediate disassembly and membrane penetration of nonenveloped viruses are not well understood.

Mammalian orthoreoviruses (reoviruses) are nonenveloped, icosahedral viruses that contain a genome consisting of 10 double-stranded RNA segments (46). Reovirus virions are formed from two concentric protein shells called outer capsid and core (46). Three reovirus serotypes are recognized based on neutralization and hemagglutination inhibition profiles. Each is represented by a prototype strain, type 1 Lang (T1L), type 2 Jones, and type 3 Dearing (T3D), which differ primarily in the sequence of viral attachment protein $\sigma 1$ (22, 45). Virtually all mammals serve as hosts for reovirus infection, but disease is restricted to the very young (58).

Reovirus infection is initiated by interactions of the $\sigma 1$ protein with one or more cell surface receptors, which include carbohydrate (5, 16, 17) and junctional adhesion molecule A (6, 10). Following attachment to cell surface receptors, reovirus enters cells by receptor-mediated endocytosis (56), which is likely to be clathrin dependent (25). Within late endosomes or lysosomes, viral outer-capsid proteins $\sigma 3$ and $\mu 1/\mu 1C$ are subject to proteolysis by endocytic proteases, resulting in generation of infectious subviral particles (ISVPs) (2, 8, 15, 52, 56). During this process, $\sigma 3$ is degraded and lost from virions, viral

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attachment protein $\sigma 1$ undergoes a conformational change, and $\mu 1/\mu 1C$ is cleaved to form particle-associated fragments $\mu 1\delta/\delta$ and ϕ (13). As the virus traverses the endocytic pathway, ISVPs are further processed to yield ISVP*s, which are characterized by conformational rearrangement of $\mu 1\delta/\delta$ and release of $\sigma 1$ (12, 14). ISVP*s penetrate endosomal membranes, releasing the transcriptionally active core particle into the cytoplasm (12, 14, 47).

Treatment of cells with the weak base ammonium chloride (AC) blocks infection by virions but not by ISVPs (19, 56), suggesting that proteolysis of $\sigma 3$ and $\mu 1/\mu 1C$ following receptor-mediated endocytosis is acid dependent. Treatment of cells with E64, an inhibitor of cysteine proteases (3), also blocks virion-to-ISVP disassembly (1), indicating that one or more cysteine proteases catalyze formation of ISVPs. In murine fibroblasts, cathepsin B and cathepsin L are acid-dependent, endosomal, cysteine proteases that mediate the disassembly of virions (23). In P388D macrophage-like cells, cathepsin S, an acid-independent, lysosomal, cysteine protease (35), serves this function for some reovirus strains in the absence of cathepsins B and L (30). Therefore, acidic pH may facilitate steps in reovirus entry by providing the appropriate conditions for the activity of the proteases resident in the endocytic compartment. Alternatively, exposure to acidic pH may trigger conformational changes in outer-capsid proteins that aid in disassembly or membrane penetration in certain cell types.

To better understand the role of acidic pH in the disassembly of reovirus virions, we selected viruses resistant to AC inhibition by serial passage of wild-type (wt) strain T3D in cells treated with AC. We tested these viruses for the capacity to grow in the presence of AC and E64, and we used reassortant genetics and nucleotide sequence analysis to determine the molecular basis for adaptation of reovirus to growth in AC-treated cells. Variant viruses were tested for susceptibility to endocytic protease cathepsin L and analyzed by cryoelectron microscopy (cryo-EM) and three-dimensional image reconstruction. The results indicate that mutations conferring AC resistance are selected in a specific region of $\sigma 3$ that likely regulates proteolytic viral disassembly.

MATERIALS AND METHODS

Cells and viruses. Murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented to contain 5% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 0.25 μ g of amphotericin per ml (Atlanta Biologicals, Atlanta, Ga.). *Spodoptera frugiperda* (Sf21) cells (Clontech, Palo Alto, Calif.) were grown in Grace's insect cell medium (Gibco Invitrogen Corp., Grand Island, N.Y.) supplemented to contain 10% fetal bovine serum, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 0.25 μ g of amphotericin per ml.

Reovirus strains T1L and T3D are laboratory stocks. Strain PI 3-1 was isolated from a persistently infected L-cell culture established using strain T3D and contains a single mutation (Y354H) in its $\sigma 3$ protein (63). Purified virion preparations were made by using second-pass (P2) L-cell lysate stocks of twice-plaque-purified reovirus as previously described (28). Viral particles were freeze-extracted from infected-cell lysates, layered onto 1.2- to 1.4-g/cm³ CsCl gradients, and centrifuged at 62,000 $\times g$ for 18 h. Bands corresponding to virions (1.36 g/cm³) were collected and dialyzed in virion storage buffer (150 mM NaCl, 15 mM MgCl₂, 10 mM Tris [pH 7.4]). Concentrations of reovirus virions in purified preparations were determined from the equivalence of 1 optical density at 260 nm and 2.1×10^{12} virions (54). ISVP preparations were made by treating 9×10^{12} particles of purified virions per ml with 0.2 mg of N- α -tosyl-L-lysine

chloromethylketone-treated α -chymotrypsin type VII from bovine pancreas (Sigma-Aldrich, St. Louis, Mo.) per ml at 37°C for 1 h.

Baculovirus vector strains were derived from *Autographa californica* nuclear polyhedrosis virus. Recombinant baculoviruses containing wt and mutant S4 gene cDNAs were generated by introduction of cDNAs into the pBacPAK8 transfer vector (BD Biosciences-Clontech), followed by recombination between transfer vector and linearized BacPAK6 *Autographa californica* nuclear polyhedrosis virus DNA (BD Biosciences-Clontech) in Sf21 cells according to the manufacturer's instructions. Recombinant viruses were isolated by plaque purification on monolayers of Sf21 cells and amplified by three passages in Sf21 cells.

Selection of reovirus variants by serial passages in the presence of AC. Independent cultures of L cells (6×10^6) in 25-cm² flasks (Corning-Costar, Acton, Mass.) were incubated for 1 h in growth medium containing 10 mM AC prior to viral adsorption. Cultures were inoculated with P2 stocks of T3D generated from independent plaque picks at a multiplicity of infection (MOI) of 10 PFU per cell. After 1 h of virus adsorption at room temperature, fresh medium containing 10 mM AC was added, and cells were incubated at 37°C for 48 h. Cultures were frozen and thawed twice, and 0.5 ml of culture lysate was used to infect a fresh culture of AC-treated L cells. This procedure was repeated for 10 passages for each of two passage series (passage series 1 and passage series 2). AC-adapted, T3D-derived (ACA-D) viruses were isolated from 10th passage lysate stocks of passage series 1 and 2 by two rounds of plaque purification on L cells maintained in the absence of AC. ACA-D1, -D2, and -D3 were isolated from the 10th passage lysate stock of passage series 1; ACA-D4, -D5, and -D6 were isolated from the 10th passage lysate stock of passage series 2. Working stocks of ACA-D viruses were prepared using L cells that were not treated with AC.

Growth of reovirus in the presence and absence of either AC or E64. Monolayers of L cells (2×10^5) in 24-well plates (Corning-Costar) were preincubated in medium supplemented to contain from 0 to 20 mM AC for 1 h or 0 to 200 μ M E64 (Sigma-Aldrich) for 4 h. The medium was removed, and cells were adsorbed with reovirus strains at an MOI of 2 PFU per cell. After incubation at 4°C for 1 h, the inoculum was removed, cells were washed with phosphate-buffered saline, and 1 ml of fresh medium supplemented with AC, from 0 to 20 mM, or E64, from 0 to 200 μ M, was added. After incubation at 37°C for 24 h, which corresponds to a single cycle of viral growth (19, 63), cells were frozen and thawed twice, and viral titers in cell lysates were determined by plaque assay (61). Independent experiments were performed using single wells of cells, which were titrated in duplicate.

Isolation and characterization of T1L $\times$ ACA-D1 reassortant viruses. Reassortant viruses were isolated as previously described (65). L-cell monolayers maintained in the absence of AC were coinfecting with reovirus strains T1L and ACA-D1 at various ratios for a total MOI of 10 PFU per cell. After development of significant cytopathic effect (approximately 48 h), putative reassortant viruses were isolated from infected cell lysates by plaque purification twice on untreated L-cell monolayers. Genotypes of reassortant viruses were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of viral double-stranded RNA purified from P2 stocks as previously described (65).

T1L $\times$ ACA-D1 reassortant viruses were tested for growth in L cells treated with and without 10 mM AC. Viral yields in both untreated and AC-treated L cells were determined for each virus by dividing the virus titer at 24 h by that at 0 h. L+AC/L ratios were calculated for the parental and reassortant viruses by dividing the average yield in the presence of AC by that in the absence of AC. An L+AC/L ratio of 1 would indicate equivalent growth in AC-treated cells and untreated cells. The reassortant viruses were ranked from highest to lowest by L+AC/L ratio, and viral gene segments that segregate with growth in the presence of AC were identified using the nonparametric Mann-Whitney test without adjustment for multiple comparisons.

The parental origin of the S4 gene segment of the reassortant viruses was confirmed by restriction digestion using PstI (New England Biolabs, Beverly, Mass.). The full-length S4 gene of each reassortant virus was amplified by reverse transcription (RT) and PCR (36) using the following primers: sense, 5'-GCTA TTTTGCCTCTTCC-3'; antisense, 5'-GATGAATGAAGCCTGTCCCACG-3'. The amplified S4 gene PCR products were treated with PstI at 37°C for 1 h, and the products were electrophoresed in an ethidium-stained 1% agarose gel. The T1L S4 gene cDNA contains a single PstI site, whereas the ACA-D1 S4 gene cDNA does not.

Nucleotide sequence analysis of ACA-D S4 genes. The $\sigma 3$ -encoding S4 gene cDNAs of six independent ACA-D viruses were generated using RT-PCR with primers specific for the noncoding regions of the T3D S4 gene as previously described (63). Resultant S4 gene cDNAs were ligated into the pCR2.1 vector (Invitrogen, San Diego, Calif.), and nucleotide sequences of cDNAs generated from at least two independent RT-PCRs were determined by automated sequencing.

Generation of ACA-S4 gene cDNAs. Site-directed mutations were introduced into the T3D S4-gene cDNA in pBacPAK 8 using PCR-based oligonucleotide mutagenesis (Stratagene) to generate ACA-D1, ACA-D2, ACA-D2a, ACA-D5, ACA-D5a, and ACA-D6 S4 gene cDNAs. Primer sets used for mutagenesis were as follows (deduced amino acid substitutions are given in parentheses and nucleotides differing from the T3D S4 sequence are underlined): ACA-D1 (I347S) sense, 5'-GCTGCTCTCACAAATGTTCCAGATACCAGCAAGTTTGGGGA T-3'; ACA-D1 (I347S) antisense, 5'-ATCCCCAAACTTGCTGTATCTGGG AACATTGTGAGAGCAGC-3'; ACA-D2a (G165H) sense, 5'-GACACTAAG CTGGATCACTACTGGACGCCTTAAAC-3'; ACA-D2a (G165H) antisense, 5'-GTTTAAGCTGTCCAGTAGTGATCCAGCTTAGTGTC-3'; ACA-D5a (I145T) sense, 5'-CACAATGTTCCAGATACCATCAAGTTTGGGGATTT GAATTATC-3'; ACA-D5a (I145T) antisense, 5'-GATAATTCAAATCCCCA AACTTGATGGTATCTGGGAACATTGTG-3'; ACA-D6 (A246G) sense, 5'-TTGGTGACGCCAGGTCGAGATTTCCGGTCACCTTGGGA-3'; ACA-D6 (A246G) antisense, 5'-TCCAAAGTGACCGAAATCTGCACCTGGCGTCAC CAA-3'. ACA-D3 and ACA-D4 S4 gene cDNAs were constructed by excising each cDNA from pCR2.1 (Invitrogen) using SacI and XbaI with subsequent ligation into linearized pBacPAK 8. Nucleotide sequences of the σ 3-encoding regions of all ACA-S4 gene cDNAs were confirmed by automated sequencing, and error-free S4 gene cDNAs were used to construct recombinant σ 3-expressing baculoviruses as previously described (64).

Expression of recombinant σ 3 proteins. Aliquots of P3 recombinant baculovirus stocks (0.5 ml) containing wt or mutant S4 gene cDNAs were used to infect Sf21 insect cells at an MOI of between 5 and 10 PFU per cell. After incubation at 25°C for 72 h, cells were scraped from the flask and centrifuged at $1,500 \times g$ for 20 min. The cell pellet was incubated on ice in 1 ml of cytoplasmic extraction buffer (10 mM Tris [pH 7.4], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol) containing 1.0 mM phenylmethylsulfonyl fluoride and an EDTA-free protease inhibitor cocktail (Roche, Indianapolis, Ind.). After 30 min of incubation, cell nuclei and membranes were collected by centrifugation at $4,450 \times g$ for 10 min. The supernatant was removed, and the cell pellet was incubated on ice in 0.8 ml of nuclear extraction buffer (20 mM Tris [pH 7.4], 0.42 M NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA) for 10 min. Cellular debris was collected by centrifugation at $16,000 \times g$ for 10 min. The supernatant was removed, analyzed by electrophoresis, and used for recoating experiments as previously described (64).

Recoating of ISVPs with expressed σ 3 proteins. ISVPs of wt T1L were incubated with recombinant T3D and ACA-D σ 3 proteins, and ISVPs of wt T3D and ACA-D viruses were incubated with recombinant T3D σ 3 protein. After incubation at 37°C for 60 to 90 min, re-coated ISVPs (rISVPs) were isolated by CsCl gradient centrifugation and analyzed by SDS-PAGE to confirm re-coating as previously described (64).

Densitometric analysis of reovirus outer-capsid proteins was used to confirm re-coating of ISVPs with σ 3 protein. Gels containing Coomassie blue-stained proteins were scanned using Photoshop Elements (Adobe Systems Incorporated, San Jose, Calif.). Mean densities were determined for bands corresponding to the σ 3 protein and either the μ 1C protein for virions or the δ protein for rISVPs using the program Scion Image Beta 3b (Scion Corporation, Frederick, Md.). The δ protein is a proteolytic cleavage product of the μ 1C protein generated during conversion of virions to ISVPs that remains particle associated (8, 15, 44, 52, 56, 62). σ 3/ μ 1C ratios were calculated for virions and compared to σ 3/ δ ratios calculated for rISVPs (64).

Treatment of reovirus virions with endocytic protease cathepsin L. Purified reovirus virions at a concentration of 1×10^{11} particles per ml in a total volume of 0.02 ml reaction buffer L (0.1 mM sodium acetate, 1 μ M EDTA, 5 mM dithiothreitol, [pH 5.5]) were treated with 0.1 mg (~0.05 units) of bovine cathepsin L (Sigma-Aldrich) per ml at 37°C for 0 to 16 h. One unit of this preparation of cathepsin L will hydrolyze 1.0 μ mol of Z-Phe-Arg-AFC per minute at pH 5.5 at 25°C (57). Protease treatment was terminated by adding 2 μ l of 10 mM E64 to the reaction mixture and freezing it at -20°C. Reaction mixtures were analyzed by SDS-PAGE.

Densitometric analysis of reovirus core protein σ 2 was used to assess σ 3 degradation during protease treatment. Gels containing Coomassie blue-stained proteins were scanned using Adobe Photoshop Elements. For each interval of protease treatment of wt and mutant virions, mean densities were determined for bands corresponding to the σ 2 and σ 3 proteins using Scion Image Beta 3b. Core protein σ 2 is not degraded during protease treatment of virions to generate ISVPs (8, 15, 24, 44, 52, 56, 62). σ 3/ σ 2 ratios were calculated, and ratios for protease-treated particles at various times of cathepsin L treatment were compared to those at time zero (24).

cryo-EM analysis of wt and ACA-D viruses. Reovirus particles were prepared for cryo-EM as previously described (21, 43). A 4- μ l aliquot of each specimen

was applied to one side of a holey carbon grid. The grid was then blotted and plunged into a bath of liquid ethane (-180°C). The frozen-hydrated sample was transferred to a precooled GATAN cryoholder (GATAN, Inc., Pleasanton, Calif.) and imaged using a JEOL 1200 transmission electron microscope (JEOL USA, Inc., Peabody, Mass.) operated at 100 kV and maintained at a specimen temperature of -163°C. Regions of interest were imaged at $\times 30,000$ magnification with an electron dose of 5 electrons per $\text{\AA}^2$. From each region, a focal pair was recorded with intended defocus values of 1 and 2 μ m. The electron microscopic images were recorded with a 1-s exposure on Kodak SO-163 film (Kodak, Rochester, N.Y.). Film was developed in Kodak D-19 developer at 21°C for 12 min and fixed in Kodak fixer at 21°C for 10 min.

Three-dimensional image reconstructions. Micrographs were selected based on particle concentration, quality of ice, and appropriate defocus conditions. Images were digitized with a Nikon Super Coolscan 9000 scanner (Nikon, Inc.) using a 6.35- μ m step size. Pixels were averaged to give a 12.7- μ m step size that corresponded to 4.23 $\text{\AA}$ per pixel in the object. Particles were boxed with an area of 280 by 280 pixels. Determination of orientational parameters, refinement of these parameters, and three-dimensional image reconstructions were performed using the ICOS Toolkit software suite (37). Orientation of the particles was determined by using the common lines approach (18) and refined by using the cross-common lines method (27). Three-dimensional image reconstructions from a set of particles that adequately represented the icosahedral asymmetric unit were computed by using cylindrical expansion methods (18). The further-from-focus micrograph in each focal pair was processed first to obtain a low-resolution reconstruction. This reconstruction was used to determine the correct orientations of particles imaged in the corresponding closer-to-focus micrograph.

Image reconstructions were computed to a resolution within the first zero of the contrast transfer function (CTF) of the corresponding micrograph. Defocus values were determined from CTF ring positions in the sum-of-particle Fourier transforms. Defocus values of various specimens in the closer-to-focus micrographs ranged from 1.0 to 1.6 μ m. Image reconstructions were corrected for the effects of the CTF using the EMAN software package (39). Final resolutions for each reconstruction were determined by Fourier ring correlation analysis (59). Image reconstructions were computed to ~24- $\text{\AA}$ resolution, which was the lowest resolution among the various specimens, for comparative analysis. Contour levels in each reconstruction were chosen to represent equal volumes between radii of ~280 $\text{\AA}$ and ~390 $\text{\AA}$. Reconstructions were viewed on a Silicon Graphics Workstation (SGI, Mountain View, Calif.) using IRIS Explorer, version 5.0, software (Numerical Algorithms Group, Inc., Oxford, United Kingdom). Further analysis of the reconstructions, volume calculations, and radial density profiles were performed using the ICOSTOOL software package (37). Two independent reconstructions, using fresh stocks of purified virions, were obtained for each virus strain.

Fitting of X-ray coordinates into cryo-EM maps. The X-ray structure of the σ 3- μ 1 complex (38) was placed into the mass density around one of the six axes using the Situs software package (66). The solutions from Situs that corresponded to the highest correlation coefficient were chosen for the fittings.

RESULTS

Selection of AC-resistant reovirus variants. To identify domains in reovirus outer-capsid proteins responsive to endocytic acidification during viral entry, two independent stocks of reovirus strain T3D were passaged serially in L cells treated with 10 mM AC (passage series 1 and 2) to select AC-resistant reovirus variants. This concentration of AC increases lysosomal pH from approximately 4.8 to 6.2 (48, 50) and inhibits reovirus growth by blocking the formation of obligatory disassembly intermediates (19, 56, 63). Following the first cycle of viral passage, 0.5 ml of culture lysate was used to infect fresh, AC-treated L-cell cultures. Cells cultivated in passage series 1 and 2 were incubated for 48 h in the presence of AC for a total of 10 passages.

To determine whether AC-resistant viruses were selected during serial passage in cells treated with AC, passage-series lysates from both passage series were tested for growth in the presence of 10 mM AC (Fig. 1). Viral titers in the presence and absence of AC were determined by plaque assay after 24 h of

FIG. 1. Selection of reovirus variants capable of growth in L cells treated with AC. Cultures of L cells (5×10^6), preincubated with 10 mM AC, were infected with independent P2 stocks of reovirus strain T3D at an MOI of 10 PFU per cell, incubated for 48 h, and then lysed by twice freezing and thawing. A 0.5-ml aliquot of culture lysate was used to infect a fresh culture of AC-treated L cells, and the process was repeated for a total of 10 passages. Monolayers of L cells (5×10^5), following a 1-h preincubation in medium with or without 10 mM AC, were infected with lysate stocks from each passage series at an MOI of 2 PFU per cell. After adsorption for 1 h, the inoculum was removed, fresh medium with or without 10 mM AC was added, and cells were incubated at 37°C for 24 h. Cells were frozen and thawed twice, and titers of virus in cell lysates were determined by plaque assay. Results for passage series 1 (A) and passage series 2 (B) are presented as mean viral titers for two independent experiments. L+AC/L ratios were calculated to assess growth in AC-treated cells relative to untreated cells. Error bars indicate the range of data.

viral growth. During serial passage, viruses in each passage series exhibited progressively higher viral titers following growth in cells treated with AC than at the first passage. To standardize for possible differences in viral replication efficiency, titers in AC-treated cells were divided by those in untreated cells to calculate L+AC/L ratios for each passage lysate (Fig. 1). We found that lysates from the 10th passage of passage series 1 and 2 displayed an approximately 40-fold and 7-fold, respectively, increase in resistance to AC compared to those derived from the first passage. These findings suggest that variant viruses altered in requirements for endocytic acidification are selected during serial passage in AC-treated cells.

Isolation of variant viruses capable of growth in the presence of AC. To facilitate studies of the mechanism of viral

FIG. 2. Effect of AC and E64 on growth of wt reovirus strains T1L and T3D and AC-adapted reovirus variants. Monolayers of L cells (2×10^5) were preincubated for either 1 h in medium with or without AC (A) or 4 h in medium with or without E64 (B) at the concentrations shown. The medium was removed, and cells were adsorbed with the virus strains shown at an MOI of 2 PFU per cell. After 1 h, the inoculum was removed, fresh medium with or without AC or E64 was added, and cells were incubated for 24 h. Viral titers in cell lysates were determined by plaque assay. The results are presented as mean viral yields, calculated by dividing the titer at 24 h by the titer at 0 h for each concentration of AC and E64, for four independent experiments. Error bars indicate standard deviations. Treatment of cells with either AC or E64 at the concentrations tested did not alter cell viability over a 24-h interval as assessed by trypan blue exclusion. Mean titers (PFU per ml) at 0 h were as follows: T1L, 4.1×10^4 ; T3D, 3.7×10^4 ; ACA-D1, 3.9×10^4 ; ACA-D2, 6.4×10^4 ; ACA-D3, 2.9×10^4 ; ACA-D4, 5.2×10^4 ; ACA-D5, 3.6×10^4 ; ACA-D6, 4.3×10^4 .

adaptation to growth in the presence of AC, we isolated six independent viral clones (ACA-D1, -D2, -D3, -D4, -D5, and -D6) from the passage series lysates. We determined yields of wt strains T1L and T3D, in vitro-generated T1L ISVPs, and each of the cloned ACA-D viruses after 24 h of growth in L cells treated with increasing concentrations of AC from 0 to 20 mM (Fig. 2A). Compared to growth in untreated cells, yields of wt T1L and T3D decreased by approximately 10-fold after growth in cells treated with 5 mM AC and approximately 300- and 600-fold, respectively, after growth in the presence of 10 mM AC. In contrast, the ACA-D variant viruses were considerably less sensitive to the inhibitory effects of AC than either strain of wt virus, producing yields in cells treated with 10 mM AC that were approximately 10-fold greater than T1L and approximately 30-fold greater than T3D (Fig. 2A). As a positive control, yields of ISVPs were decreased by only approxi-

TABLE 1. Growth of T1L $\times$ ACA-D1 reassortant viruses in AC-treated cells

Virus isolate	Origin of gene segment ^a										Mean L+AC/L ratio (SE) ^b
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4 ^c	
Parental											
T1L	L	L	L	L	L	L	L	L	L	L	0.035 (0.012)
ACA-D1	D	D	D	D	D	D	D	D	D	D	0.090 (0.032)
Reassortant											
43	D	D	D	L	L	L	D	D	D	D	0.402 (0.052)
9	D	D	L	D	L	D	L	D	D	D	0.149 (0.051)
17	D	D	D	D	D	D	L	D	D	D	0.127 (0.052)
118	D	D	L	D	D	D	D	D	D	D	0.104 (0.046)
111	L	D	L	L	L	L	L	L	L	L	0.078 (0.017)
105	D	D	D	L	D	D	D	D	D	L	0.079 (0.006)
102	L	D	L	D	L	D	L	D	L	D	0.076 (0.001)
130	L	D	L	L	D	L	L	L	D	L	0.063 (0.001)
104	D	D	D	D	D	D	D	D	D	L	0.059 (0.011)
38	L	L	L	L	D	L	L	D	L	L	0.029 (0.006)

^a Parental origin of each gene segment: L, gene segment derived from T1L; D, gene segment derived from ACA-D1.

^b L cells (2×10^5) were adsorbed with virus strains at an MOI of 2 PFU per cell. After 1 h, the inoculum was removed, fresh medium with or without 10 mM AC was added, and cells were incubated at 37°C for 24 h. Cells were frozen and thawed twice, and virus titers were determined by plaque assay using L cell monolayers. The viral yield in AC-treated cells was divided by that in untreated L cells to calculate an L+AC/L ratio for each virus isolate. Growth assays were performed in duplicate. Shown are the mean L+AC/L ratios and standard error (SE) values.

^c The S4 gene was the only gene associated with growth in AC-treated cells as determined by using the nonparametric Mann-Whitney test, without adjusting for multiple comparisons ($P = 0.032$).

mately fivefold after infection of cells treated with the highest concentration of AC tested, 20 mM. Therefore, AC-resistant reovirus variants suitable for use in studies of acid-dependent reovirus disassembly are selected during serial passage in L cells treated with AC.

Growth of ACA-D viruses in cells treated with E64. To test whether the ACA-D viruses are also resistant to growth inhibitory effects of the cysteine protease inhibitor E64, we determined yields of wt T1L and T3D, T1L ISVPs, and the ACA-D viruses after 24 h of growth in L cells treated with increasing concentrations of E64 from 0 to 200 μ M (Fig. 2B). Yields of T1L and T3D were decreased by approximately 20- and 200-fold, respectively, in cells treated with 50 μ M E64 and approximately 100- and 1,000-fold, respectively, in cells treated with 100 μ M E64. Similar to findings made in experiments using AC-treated cells, ISVPs were more resistant to inhibition by E64, with yields decreased by less than 30-fold after infection of cells treated with the highest concentration tested, 200 μ M E64. Following growth in the presence of 100 μ M E64, yields of all six ACA-D variant viruses were greater than that produced by wt T3D. ACA-D3 displayed the greatest resistance to E64 inhibition among the ACA-D viruses, with an approximately 100-fold decrease in yield in cells treated with 100 μ M E64 compared to an approximately 150-fold to 500-fold decrease for the other ACA-D viruses. Thus, all of the ACA-D variants are more resistant than wt parental T3D to both AC and E64, although the extent of resistance to AC appears to be greater than that to E64.

Identification of viral genes that segregate with ACA-D virus growth in the presence of AC. To determine mechanisms by which mutations selected during serial passage of reovirus in AC-treated cells alter the requirement for acidification during viral entry, we used reassortant genetics to identify viral genes associated with growth of ACA-D viruses in the presence of AC. Reassortant viruses were isolated from crosses of wt T1L and ACA-D1, and the parental origin of the progeny virus

gene segments was determined by SDS-PAGE (Table 1). T1L $\times$ ACA-D1 reassortant viruses were tested for growth in the presence and absence of 10 mM AC. This concentration of AC was chosen to maximize differences in growth between wt and ACA-D viruses (Fig. 2A). Viral yields were determined after 24 h by dividing viral titer at 24 h by that at 0 h. L+AC/L ratios were calculated for each reassortant virus by dividing the viral yield in AC-treated cells by that in untreated cells (Table 1). Reassortant viruses were ranked from highest to lowest by L+AC/L ratio. Using the Mann-Whitney test, only the S4 gene, which encodes the $\sigma 3$ protein, was associated with growth in AC-treated cells ($P = 0.032$). Reassortant viruses with the four highest L+AC/L ratios, ranging from 0.104 to 0.402, had an S4 gene derived from ACA-D1, whereas viruses with the three lowest ratios, ranging from 0.029 to 0.063, had an S4 gene derived from wt T1L. The parental origin of the S4 gene of the reassortant viruses was confirmed by restriction digestion with PstI, which digests the S4 gene cDNA of T1L but not that of ACA-D1 (data not shown). Therefore, these results suggest that mutations in the $\sigma 3$ -encoding S4 gene selected during passage of reovirus in AC-treated cells determine the capacity of ACA-D viruses to generate higher viral yields than wt virus after infection of cells treated with AC.

S4 gene nucleotide sequences of ACA-D reovirus variants.

To identify mutations associated with the capacity of ACA-D viruses to infect cells in the presence of AC, we determined the S4 gene nucleotide sequences of the six independently derived ACA-D viruses. The S4 gene of reovirus T3D is 1,196 nucleotides in length and encodes the 365-amino-acid $\sigma 3$ protein in a single open reading frame (29). RT-PCRs using oligonucleotide primers complementary to the 5' and 3' untranslated regions of the S4 gene were used to generate cDNA clones corresponding to full-length coding regions of the S4 gene of the six ACA-D virus strains (Table 2). Each of the S4 genes contained one or two nucleotide mutations, which resulted in one or two unique amino acid substitutions in the sequence of

TABLE 2. Mutations in S4 gene nucleotide sequences of ACA-D viruses and corresponding mutations in deduced amino acid sequences of their $\sigma 3$ proteins

Virus strain	Location and nature of mutation in: ^a		GenBank accession no.
	S4 gene	$\sigma 3$ protein	
ACA-D1	1072, U → G	347, Ile → Ser	DQ004470
ACA-D2	526, G → C	165, Gln → His	DQ004471
	769, C → G	246, Ala → Gly	
ACA-D3	1092, U → C	354, Tyr → His	DQ004472
ACA-D4	571, U → C	180, Ile → Thr	DQ004473
ACA-D5	466, U → C	145, Ile → Thr	DQ004474
	1072, U → G	347, Ile → Ser	
ACA-D6	769, C → G	246, Ala → Gly	DQ004475
D2a ^b	526, G → C	165, Gln → His	
D5a ^b	466, U → C	145, Ile → Thr	

^a Mutations in the S4 gene refer to nucleotide position; mutations in the $\sigma 3$ protein refer to amino acid position.
^b D2a and D5a $\sigma 3$ proteins were generated by site-directed mutagenesis of ACA-D2 and ACA-D5 S4 gene cDNAs, respectively.

$\sigma 3$. Four of the mutations (I180T, A246G, I347S, and Y354H) are in close proximity in the crystal structure of the protein (49) (Fig. 3) and are near sites cleaved by the endocytic protease cathepsin L (23) (Fig. 3B). The single mutation in ACA-D3 $\sigma 3$, Y354H, has been selected previously during persistent infection of L cells by T3D (63) and by serial passage of T3D in L cells treated with E64 (24). Viruses containing this

mutation in $\sigma 3$ are resistant to both AC and E64 and display enhanced susceptibility to proteolysis (24, 63, 64). Thus, serial passage of reovirus strain T3D in the presence of AC selects several novel mutations in $\sigma 3$ as well as a mutation known to alter the disassembly of reovirus virions, Y354H.

Recoating ISVPs with wt and ACA-D $\sigma 3$ proteins. To determine whether the mutations in the $\sigma 3$ protein selected during serial passage in AC-treated L cells are sufficient to confer growth in the presence of AC and to assess the contribution of other possible mutations selected during serial passage to the AC resistance phenotype, we generated recombinant baculoviruses to express wt and mutant $\sigma 3$ proteins for use in recoating studies (Table 2). The $\sigma 3$ proteins of T3D, ACA-D1, -D3, -D4, and -D6, along with two additional mutant $\sigma 3$ proteins containing the single unique amino acid substitutions in ACA-D2 and ACA-D5, D2a and D5a, respectively, were used in these experiments. Insect cells were infected with recombinant baculoviruses, and nuclear extracts were prepared. Protein expression was verified by SDS-PAGE, in which the major protein band in nuclear extracts migrated with the electrophoretic mobility of native $\sigma 3$ protein (data not shown).

To generate recoated T1L particles, T1L ISVPs were incubated with recombinant $\sigma 3$ proteins expressed in Sf21 cells. After incubation, rISVPs were isolated by CsCl gradient centrifugation and analyzed by SDS-PAGE (Fig. 4A). Each of the recombinant $\sigma 3$ proteins tested was capable of recoating T1L

FIG. 3. Mutations in $\sigma 3$ protein of ACA-D viruses. (A) Ribbon diagram of the 1.8-Å crystal structure of the T3D $\sigma 3$ protein (49). The figure is rotated by two turns of 45° to indicate the location of unique mutations in the ACA-D viruses (D1 through D6). Mutations found in ACA-D virus $\sigma 3$ proteins are I145R (orange), Q165H, (green), I180T (yellow), A246G (purple), I347S (blue), and Y354H (red). (B) The virion-distal lobe of $\sigma 3$ is enlarged to highlight side chains of residues mutated in the ACA-D viruses. Cathepsin L cleaves $\sigma 3$ between amino acids 243/244 and 250/251 (23) (black in the α -carbon tracing and indicated by arrows).

FIG. 4. ISVPs recoated with recombinant $\sigma 3$ proteins. ISVPs of either reovirus strain T1L (A) or the ACA-D viruses shown (B) were incubated with insect cell nuclear extracts containing the indicated $\sigma 3$ proteins to generate rISVPs. Equal numbers of rISVPs were analyzed by SDS-PAGE with virions and ISVPs used as controls. Viral proteins are labeled on the left. Densities of bands corresponding to $\sigma 3$ and either $\mu 1C$ protein for virions or δ protein for ISVPs were determined by using the program Scion Image Beta 3b. $\sigma 3/\mu 1C$ or $\sigma 3/\delta$ ratios are indicated at the bottom of the gels.

ISVPs, resulting in the generation of rT3D, rD1, rD2a, rD3, rD4, rD5a, and rD6. To assess the stoichiometry of $\sigma 3$ protein in the recoated particles, the densities of bands corresponding to $\sigma 3$ were compared to those of $\mu 1C$ cleavage product δ (Fig. 4). For each rISVP species, the $\sigma 3/\delta$ ratio approximated that of the $\sigma 3/\mu 1C$ ratio found in virions, approximately 1:1. These results demonstrate that recombinant $\sigma 3$ proteins recoat ISVPs with approximately native stoichiometry.

Growth of rISVPs in the presence and absence of AC. To confirm that the mutations in ACA-D $\sigma 3$ proteins confer viral growth in the presence of AC, virions and rISVPs were tested for the capacity to infect AC-treated cells. L cells were infected with T1L virions, T1L ISVPs, or rISVPs generated by using the mutant $\sigma 3$ proteins with the single amino acid substitutions shown in Table 2 in the presence or absence of 10 mM AC. After 24 h of growth, viral titers in cell lysates were determined by plaque assay (Fig. 5A). Each of the particles tested produced approximately equivalent yields after 24 h of growth in the absence of AC treatment. However, in the presence of AC, ISVPs recoated with ACA-D1, -D3, -D4, and -D6 $\sigma 3$ proteins produced approximately 10-fold-greater yields than those recoated with T3D, D2a, and D5a $\sigma 3$ proteins. Thus, four of the

FIG. 5. Growth of rISVPs in the presence and absence of AC. Monolayers of L cells (2×10^5) were preincubated for 1 h in medium supplemented with or without 10 mM AC. The medium was removed, and cells were adsorbed with either wt T1L ISVPs recoated with ACA-D $\sigma 3$ (A) or ACA-D ISVPs recoated with wt T3D $\sigma 3$ (B) at an MOI of 2 PFU per cell. After 1 h, the inoculum was removed, fresh medium with or without AC was added, and cells were incubated for 24 h. Viral titers in cell lysates were determined by plaque assay. The results are presented as mean viral yields, calculated by dividing the titer at 24 h by the titer at 0 h, for two independent experiments. Error bars indicate the range of data.

six mutations in the $\sigma 3$ protein selected during serial passage in AC-treated cells, which are the four mutations that cluster in the virion-distal lobe of the protein (Fig. 3), confer resistance to the growth inhibitory effects of AC.

To determine whether mutations in the ACA-D virus $\sigma 3$ proteins are sufficient to produce the AC resistance phenotype, ISVPs of each of the six ACA-D variant viruses were recoated with wt T3D $\sigma 3$ protein, analyzed by SDS-PAGE (Fig. 4B), and tested for growth in cells treated with 10 mM AC (Fig. 5B). ACA-D ISVPs recoated with T3D $\sigma 3$ produced yields equivalent to or less than T3D ISVPs recoated with T3D $\sigma 3$. Therefore, we conclude that mutations in the $\sigma 3$ protein selected during serial passage in AC-treated cells determine resistance to AC-mediated growth inhibition.

Treatment of wt and ACA-D reoviruses with endocytic protease cathepsin L. To test whether the resistance to AC displayed by the ACA-D viruses correlates with altered susceptibility to protease treatment in vitro, virions of wt T3D, PI 3-1, and the ACA-D viruses with the single, unique mutations in $\sigma 3$ (Table 2) were treated with the endocytic protease cathepsin L. PI 3-1 has a single mutation in $\sigma 3$ (Y354H) that confers en-

FIG. 6. (A) Electrophoretic analysis of reovirus structural proteins during treatment of virions with cathepsin L. Purified virions of reovirus strains T3D, PI 3-1, and the ACA-D viruses shown were treated with bovine cathepsin L (pH 5.5) for the indicated intervals. Equal numbers of viral particles (1×10^{11} particles) were loaded into wells of 10% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue. Viral proteins are labeled on the left. The results shown are representative of two experiments performed for each virus. (B) Quantitation of $\sigma 3$ band intensity following treatment with cathepsin L. Densities of bands corresponding to the $\sigma 3$ and $\sigma 2$ proteins were determined by using the program Scion Image Beta 3b. The results are presented as the mean $\sigma 3/\sigma 2$ ratios for two independent experiments.

hanced susceptibility of the virus to proteolytic disassembly (63). Cathepsin L is a cysteine protease that digests reovirus virions to form functional ISVPs (2, 23). Virions were treated over a time course from 0 to 16 h with purified, bovine cathepsin L at pH 5.5, which is the pH optimum for this enzyme (4), and viral structural proteins were analyzed by SDS-PAGE (Fig. 6A). Treatment of wt T3D and PI 3-1 virions with cathepsin L resulted in proteolytic digestion of outer-capsid proteins indicative of ISVP formation, with degradation of $\sigma 3$ and cleavage of $\mu 1C$ to form δ . In comparison to T3D, proteolysis of PI 3-1 during cathepsin L treatment occurred with substantially faster kinetics, as previously reported (64). Treatment of the ACA-D virions with cathepsin L also yielded proteolytic digestion of $\sigma 3$ and cleavage of $\mu 1C$ to δ but with kinetics for three of the four ACA-D viruses tested that were intermediate to those of T3D and PI 3-1 (Fig. 6A). Densitometric analysis of $\sigma 3/\sigma 2$ band intensities for PI 3-1 and ACA-D3, which have identical mutations in $\sigma 3$ (63), demonstrated that >90% of $\sigma 3$ was removed from the viral outer capsid by 2 h of treatment

FIG. 7. Three-dimensional image reconstructions of T3D and ACA-D virions. cryo-EM reconstructions viewed along an icosahedral three-fold axis of wt T3D (A), ACA-D1 (B), ACA-D4 (C), and ACA-D6 (D) are shown.

with cathepsin L using these experimental conditions. In contrast, cathepsin L treatment of the other three ACA-D viruses yielded complete removal of $\sigma 3$ after 4 h of incubation for ACA-D1 and ACA-D6 and after 8 h of incubation for ACA-D4, whereas T3D $\sigma 3$ was still evident after 16 h of incubation (Fig. 6B). Thus, digestion of ACA-D virions with cathepsin L occurs more rapidly than digestion of wt virions. However, with the exception of ACA-D3, none of the ACA-D viruses are digested as rapidly as PI 3-1.

Cryo-EM structures of wt and ACA-D virions. To determine whether the resistance to AC exhibited by the ACA-D viruses is associated with structural alterations of the viral outer capsid, cryo-EM and three-dimensional image analysis was used to compare the structures of wt T3D and ACA-D1, -D4, and -D6. Virions of T3D (computed to 24-Å resolution with 162 particles) and ACA-D1, -D4, and -D6 (computed to 24-Å resolution with 102, 116, and 164 particles, respectively) have similar morphological characteristics (Fig. 7). In each of the reconstructions, the fingerlike projections of $\sigma 3$ are organized on an incomplete $T = 13$ icosahedral lattice with six $\sigma 3$ subunits at the local 6-fold axes and an incomplete ring of four $\sigma 3$ subunits adjacent to the 5-fold axes surrounding the $\lambda 2$ turret. These results demonstrate that both the overall arrangement of $\sigma 3$ protein and protein-protein interactions in the outer capsid of wt and ACA-D virions are similar.

To determine whether structures of wt and ACA-D $\sigma 3$ proteins differ, we examined cross-sectional density slices at 5-Å intervals through $\sigma 3$ molecules perpendicular to a 6-fold axis from each of the reconstructions (data not shown). For this analysis, the reconstructions were radially scaled to match the peak corresponding to the innermost ($\lambda 1$) layer. Density slices at radii corresponding to the $\sigma 3$ protein, including regions that contain the mutations in the ACA-D viruses, are equivalent, with all of the $\sigma 3$ monomers having similar shape and connec-

tivity. These findings, together with placement of the X-ray coordinates of the $\sigma 3$ - $\mu 1$ complex (38) into the cryo-EM images (data not shown), suggest that the ACA-D $\sigma 3$ proteins are structurally indistinguishable from wt T3D $\sigma 3$ at the resolution of this analysis.

DISCUSSION

In this study, we show that AC-resistant reovirus variants are selected by serial passage in murine L cells treated with AC. After growth in cells treated with 10 mM AC, yields of six independently derived AC-adapted viral variants are 30-fold greater than those of wt parental strain T3D. Growth of the ACA-D variants in the presence of AC segregates with the S4 gene, which encodes outer-capsid protein $\sigma 3$. Notably, the relevant mutations in the ACA-D virus $\sigma 3$ proteins cluster in the virion-distal lobe of the protein. These results suggest that a discrete region in $\sigma 3$ influences the susceptibility of reovirus to growth inhibition by AC.

Although analysis of T1L $\times$ ACA-D1 reassortant viruses indicates that resistance to AC is determined primarily by mutations in the S4 gene (Table 1), it is possible that other viral genes contribute to the growth differences exhibited by these reassortant viruses in AC-treated cells. Viral yields are influenced by many viral replication steps subsequent to cell entry, and viral genes that influence these steps might act to moderate differences in growth of reassortants that have altered entry phenotypes (24). Therefore, it is not surprising that the L-plus-AC/L ratios observed for the T1L $\times$ ACA-D1 reassortant viruses in this study form a continuum. However, using the nonparametric Mann-Whitney test, the S4 gene was the only viral gene associated with the differences in L+AC/L ratios exhibited by the T1L $\times$ ACA-D1 reassortant viruses. Therefore, the S4 gene likely plays the dominant role in determining the AC resistance phenotype.

We directly tested whether mutations in the ACA-D S4 genes confer resistance to AC by comparing T1L ISVPs recoated with either wt or mutant $\sigma 3$ proteins for growth in AC-treated cells. We found that four of the mutations selected during serial passage in the presence of AC, I180T, A246G, I347S, and Y354H, are responsible for the AC resistance displayed by the ACA-D viruses (Fig. 5A). Importantly, ACA-D ISVPs and T3D ISVPs recoated with wt $\sigma 3$ do not differ in AC-mediated growth inhibition (Fig. 5B), indicating that ACA-D capsid proteins other than $\sigma 3$ do not confer AC resistance in the absence of ACA-D $\sigma 3$. Of the four unique mutations selected, three are novel. The Y354H mutation has been selected previously during persistent infection (PI viruses) (63) and by serial passage in L cells treated with E64 (D-EA viruses) (24).

Both PI viruses and D-EA viruses exhibit enhanced kinetics of disassembly with degradation of $\sigma 3$ and cleavage of $\mu 1C$ occurring much more rapidly both in vitro and in cells (24, 63). cryo-EM image reconstructions of virions of PI viruses indicate that the Y354H mutation is associated with an alteration in $\sigma 3$ structure at the hinge region between the two lobes of the protein (64). These findings suggest that the C-terminal region of $\sigma 3$ encompassing residue 354 regulates susceptibility of the protein to cleavage. This region also has been shown to dictate strain-specific differences in the susceptibility of $\sigma 3$ to proteo-

lytic attack (33, 34). The $\sigma 3$ protein of strain T1L is cleaved with faster kinetics than that of T3D. Analysis of ISVPs recoated with chimeric $\sigma 3$ proteins generated from T1L and T3D indicates that the C-terminal region of $\sigma 3$ is primarily responsible for the rate of $\sigma 3$ proteolysis. Moreover, sequence polymorphisms at residues 344, 347, and 353 in $\sigma 3$ are the primary determinants of the difference in cleavage susceptibility exhibited by T1L and T3D $\sigma 3$ (33). Thus, the new mutants reported here, coupled with previous studies of viruses with altered disassembly kinetics, point to a critical role for sequences in the virion-distal lobe of $\sigma 3$ in influencing susceptibility to acid-dependent proteolysis.

Cathepsin L is the principle endocytic protease that mediates reovirus disassembly in fibroblasts (23). ACA-D viruses are cleaved more rapidly than wt virus by cathepsin L (Fig. 6), suggesting that variant viruses selected during growth in AC-treated cells are altered in their sensitivity to proteolysis. Susceptibility of $\sigma 3$ to proteolytic cleavage during generation of ISVPs is regulated by sequences in a discrete region of the protein termed the "protease-hypersensitive region," or HSR (33). The HSR in $\sigma 3$ is strain specific, corresponding to residues 238 to 242 in T1L and 208 to 214 in T3D, and encompasses cleavage sites for several proteases, including cathepsin L and intestinal proteases chymotrypsin and trypsin. Residue 180 in T3D $\sigma 3$, which is altered in ACA-D4, has been proposed to influence both the structure of the HSR and the cleavage rate of $\sigma 3$ (33). Residue 347 in T3D $\sigma 3$, which is altered in ACA-D1 and ACA-D5, is one of three C-terminal residues thought to be responsible for differences in both the site used for cleavage and the rate of cleavage exhibited by T1L and T3D $\sigma 3$ (33). Residue 246, which is altered in ACA-D2 and ACA-D6, and residue 354, which is altered in ACA-D3, are also located in a region that has been suggested to be important for regulating access to the HSR and influencing cleavage kinetics (33). Therefore, mutation of any of these residues could plausibly affect $\sigma 3$ cleavage, perhaps by influencing access to the HSR.

cryo-EM and three-dimensional image analyses revealed that ACA-D virions contain a full complement of $\sigma 3$ proteins, with similar overall arrangements of $\sigma 3$ and similar protein-protein interactions within the outer capsid (Fig. 7). This observation suggests that the diminished sensitivity to growth inhibition by AC and the enhanced susceptibility to proteolysis of the ACA-D viruses are not due to defects in viral assembly. We did not detect differences in the structure of ACA-D $\sigma 3$ proteins in comparison to wt T3D $\sigma 3$ at the resolution of the analysis performed in this study. This finding suggests that the ACA-D mutations in $\sigma 3$ do not lead to major structural alterations. However, it is possible that these mutations cause subtle alterations affecting accessibility to the protease cleavage site. If so, detection of such alterations will require higher-resolution structural analysis.

How might the mutations selected during serial passage in AC-treated cells confer an enhanced capacity of reovirus to grow in the presence of AC? We think that there are two possibilities. First, mutations in ACA-D $\sigma 3$ may mimic an acid-induced conformational change in the protein that is generated upon entry of virions into the endocytic pathway. Such an acid-induced conformational change may promote protease access to the HSR and increase susceptibility to cleavage. Sec-

ond, mutations in ACA-D $\sigma 3$ may impart enhanced susceptibility to proteolysis by either generating additional protease cleavage sites or by causing local structural alterations that render the internal cleavage sites more susceptible to protease, independent of any acid-induced conformational change. Our data do not allow us to definitively distinguish between these possibilities. However, since maximum cathepsin L activity occurs at acidic pH, AC resistance mutations in ACA-D virus $\sigma 3$ proteins may allow proteolysis by this enzyme even when its activity is attenuated by AC.

It is noteworthy that, with the exception of ACA-D3, the ACA-D viruses reported here display resistance to AC and E64 (Fig. 2) and kinetics of proteolytic disassembly (Fig. 6) intermediate to those of wt viruses and mutant PI (63) and D-EA (24) viruses. Like ACA-D3, PI and D-EA viruses have a Y354H mutation in $\sigma 3$ (24, 63, 64). His354 in $\sigma 3$ is associated with structural alterations in the protein that are detectable by cryo-EM and three-dimensional image processing (64) at the resolution of the structural analysis employed in our study. Therefore, the biological and biochemical data correlate with the structural analysis and suggest that the ACA-D mutations confer more modest alterations in disassembly properties than the previously reported mutants with a histidine at position 354 in $\sigma 3$ (24, 63, 64). These differences may be caused by differences in the magnitude of the underlying structural alterations associated with the different $\sigma 3$ mutations.

Proteases other than cathepsin B and cathepsin L are also capable of removing $\sigma 3$ during viral disassembly. In P388D cells, a macrophage-like cell line, cathepsin S mediates uncoating of some reovirus strains in the absence of cathepsins B and L (30). Reovirus infection of P388D cells is not blocked by AC, indicating that viral entry into these cells does not require acidic pH. However, viral yields in P388D cells are substantially less after infection by virions than by ISVPs (30). This result suggests that virion disassembly in these cells is not optimally productive, perhaps due to inefficient proteolysis mediated by cathepsin S. Therefore, it is possible that the low-pH environment of some cell types enhances the disassembly process.

Viral disassembly is an irreversible process that must be highly coordinated with a permissive cellular environment to allow productive infection. Interestingly, viruses with alterations in disassembly kinetics are often attenuated in vivo. PI viruses (including those with the Y354H mutation in $\sigma 3$) display delayed clearance following intracranial inoculation of newborn mice (42). However, none of the PI viruses tested are more virulent than wt virus as assessed by 50% lethal dose values (42). It will be interesting to examine the virulence and pathogenesis of the ACA-D viruses reported here. Given the alterations in the requirement for acidic pH to achieve productive viral entry, these viruses may exhibit altered tropism and virulence.

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