Reovirus Variants Selected during Persistent Infections of L Cells Contain Mutations in the Viral S1 and S4 Genes and Are Altered in Viral Disassembly

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Reoviruses isolated from persistently infected cultures (PI viruses) can grow in the presence of ammonium chloride, a weak base that blocks acid-dependent proteolysis of viral outer-capsid proteins during viral entry into cells. We used reassortant viruses isolated from crosses of wild-type (wt) reovirus strain, type 1 Lang, and three independent PI viruses, L/C, PI 2A1, and PI 3-1, to identify viral genes that segregate with the capacity of PI viruses to grow in cells treated with ammonium chloride. Growth of reassortant viruses in ammonium chloride-treated cells segregated with the S1 gene of L/C and the S4 gene of PI 2A1 and PI 3-1. The S1 gene encodes viral attachment protein $\sigma 1$, and the S4 gene encodes outer-capsid protein $\sigma 3$. To identify mutations in $\sigma 3$ selected during persistent reovirus infection, we determined the S4 gene nucleotide sequences of L/C, PI 2A1, PI 3-1, and four additional PI viruses. The deduced amino acid sequences of $\sigma 3$ protein of six of these PI viruses contained a tyrosine-to-histidine substitution at residue 354. To determine whether mutations selected during persistent infection alter cleavage of the viral outer capsid, the fate of viral structural proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after treatment of virions of wt and PI viruses with chymotrypsin *in vitro*. Proteolysis of PI virus outer-capsid proteins $\sigma 3$ and $\mu 1C$ occurred with faster kinetics than proteolysis of wt virus outer-capsid proteins. These results demonstrate that mutations in either the S1 or S4 gene alter acid-dependent disassembly of the reovirus outer capsid and suggest that increased efficiency of proteolysis of viral outer-capsid proteins is important for maintenance of persistent reovirus infections of cultured cells.

For cytolytic viruses to maintain persistent infections of cultured cells, interactions between virus and cell must be fundamentally altered such that a less cytopathic virus-host relationship is established (reviewed in reference 4). Either variant viruses that are altered in cytolytic potential or variant cells that are less permissive for viral replication may be selected to facilitate maintenance of persistent infection. In some cases, however, viruses and cells coevolve during persistent infection, such that selection of virus-resistant cells leads to counterselection of mutant viruses that can infect the resistant cells (4). Studies of these coevolving cultures of viruses and cells have identified key steps in virus-cell interaction that are modified by cells to resist cytolytic infection. Specifically, steps in viral replication required for viral entry are targeted for mutations in several types of persistent infections, including those caused by coronavirus (13, 19), poliovirus (10, 21), and reovirus (16, 41). Identification of specific strategies used by viruses to overcome cellular blocks to viral entry will increase our understanding of mechanisms used by viruses to enter cells.

Reovirus enters cells by receptor-mediated endocytosis (8, 9, 33, 38). Within late endosomes or lysosomes, viral outer-capsid proteins $\sigma 3$ and $\mu 1/\mu 1C$ are subject to proteolysis by cellular proteases, resulting in generation of infectious subviral particles (ISVPs) (9, 12, 36, 38). During this process, $\sigma 3$ is degraded and lost from virions, viral attachment protein $\sigma 1$ undergoes a conformational change, and $\mu 1/\mu 1C$ is cleaved to

form the particle-associated fragments $\mu 1\delta/\delta$ and ϕ (reviewed in reference 31). ISVPs generated in the endocytic compartment are likely to be identical to those generated either in the intestinal lumen of the infected host (5, 7) or *in vitro* by digestion of virions with chymotrypsin or trypsin (9, 12, 27, 36, 38). Treatment of cells with the weak base ammonium chloride (16, 38) or inhibitors of the vacuolar proton ATPase, such as bafilomycin or concanamycin A (24), blocks infection by virions but not by ISVPs. These findings suggest that proteolysis of $\sigma 3$ and $\mu 1/\mu 1C$ is acid dependent. Entry steps are completed by interactions of ISVPs with vacuolar membranes, leading to delivery of transcriptionally active cores into the cytoplasm (20, 29, 30).

During persistent reovirus infections, mutations that affect viral entry are selected in viruses and cells (16, 41). When persistently infected cultures of murine L929 (L) cells (16) or murine erythroleukemia cells (41) are cured of persistent infection by treatment with antireovirus antibodies, the resulting cells do not fully support growth of wild-type (wt) viruses. However, when virions of wt viruses are first converted to ISVPs by chymotrypsin treatment *in vitro*, cured cells support good growth of wt viruses (16, 41). These findings indicate that cellular mutations selected during persistent reovirus infection likely affect proteolytic processing of viral outer-capsid proteins. In contrast to wt viruses, viruses isolated from persistently infected cultures (PI viruses) can grow in L cells treated with ammonium chloride (16, 41). These results suggest that selection of mutant cells that do not support virion-to-ISVP processing leads to selection of mutant viruses that have altered requirements for decreased pH to complete steps in viral entry. Mechanisms by which mutations in PI viruses promote

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TABLE 1. Prototype PI viruses isolated from independent persistently infected cell cultures

PI virus strain ^a	Day of harvest ^b	Persistently infected culture	hp stock	AC/L ratio ^c
L/C	281	L/C	<i>ts</i> C447	0.13
PI 2A1	151	L/T3D-2A	T3D-2	0.15
PI 3-1	178	L/T3D-3	T3D-3	0.32
L/T2 + C	304	L/T2 + C	wt T2J + hp <i>ts</i> C447	0.24
L/T2 + T3	294	L/T2 + T3	wt T2J + hp T3D	0.19
PI 1A1	151	L/T3D-1A	T3D-1	0.21
PI 5-1	89	L/T3D-5	T3D-5	0.56

^a L/C virus was isolated from a persistently infected L-cell culture initiated with T3D mutant *ts*C447, which had been passaged serially at a high MOI (3). L/T2 + C and L/T2 + T3 were derived from persistently infected L-cell cultures initiated by coinfection of cells with T2J and a high-passage (hp) stock of *ts*C447 and T2J and a high-passage stock of T3D, respectively (2). The remaining PI viruses were isolated from persistently infected L-cell cultures initiated with independent high-passage stocks of T3D (16).

^b Day of culture that the PI virus strain was isolated.

^c L cells (5×10^5) were infected with virus strains at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium with or without 10 mM ammonium chloride was added, and the cells were incubated at 37°C for 24 h. Cells were frozen and thawed twice, and virus in cell lysates was titrated on L-cell monolayers by plaque assay. Experiments were performed in duplicate, and the mean viral yield in L cells treated with ammonium chloride was divided by that in untreated L cells to calculate an AC/L ratio for each virus strain. The AC/L ratio for wt strain T3D was 0.0078.

decreased requirements for acidic pH to facilitate entry steps are not known.

In this study, we used reovirus reassortant genetics to identify viral genes that segregate with growth of PI viruses in the presence of ammonium chloride. The S4 gene nucleotide sequences of seven independent PI viruses were determined to identify mutations in $\sigma 3$ selected during persistent infection. The effect of viral mutations on virion disassembly was investigated by determining the fate of viral outer-capsid proteins during chymotrypsin treatment of virions to generate ISVPs in vitro. Our results demonstrate that mutations in two viral outer-capsid proteins, $\sigma 1$ and $\sigma 3$, are selected during persistent infection and suggest that these mutations enhance virus growth in mutant cells by altering susceptibility of outer-capsid proteins to proteolysis during viral entry.

MATERIALS AND METHODS

Cells and viruses. 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 (Intergen, Purchase, N.Y.), 2 mM L-glutamine, and 100 U of penicillin, 100 μ g of streptomycin, and 250 ng of amphotericin per ml (Irvine). Reovirus strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) are laboratory stocks. PI viruses L/C, PI 2A1, PI 3-1, L/T2 + C, L/T2 + T3, PI 1A1, and PI 5-1 were isolated previously from independent persistently infected L-cell cultures (2, 3, 16) (Table 1). T1L $\times$ L/C (22), T1L $\times$ PI 2A1 (42), and T1L $\times$ PI 3-1 (42) reassortant viruses were isolated previously. Purified virion preparations were made by using second-passage L-cell lysate stocks of twice-plaque-purified reovirus as previously described (18).

Growth of reovirus in the presence and absence of ammonium chloride. Monolayers of L cells (2×10^5) were infected with reovirus strains at a multiplicity of infection (MOI) of 2 PFU per cell in 24-well plates (Costar, Cambridge, Mass.). After a 1-h adsorption period at 4°C, the inoculum was removed, cells were washed twice with phosphate-buffered saline, and 1.0 ml of fresh medium supplemented to contain either 0 or 10 mM ammonium chloride was added. After incubation at 37°C for defined intervals, cells were frozen and thawed twice, and virus in cell lysates was titrated on L-cell monolayers by plaque assay (39). Independent experiments were performed with single wells of cells, which were titrated in duplicate.

Statistical analysis. The association of reovirus gene segments with growth in L cells treated with ammonium chloride was determined by the nonparametric Mann-Whitney test, using Minitab (release 8) statistical software (Addison-Wesley, Reading, Mass.).

Sequencing PI virus S4 genes. For PI viruses L/C, PI 2A1, PI 3-1, PI 1A1, and PI 5-1, genomic S4 gene double-stranded RNA (dsRNA) was purified from virions and sequenced by using dideoxy sequencing reactions with oligodeoxynucleotide primers, reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and [³⁵S]dATP (1,000 Ci per mmol; Amersham, Arlington Heights, Ill.) according to previously described techniques (6, 28). Full-length sequences of the coding strand were determined for each PI virus S4 gene; sequence changes were confirmed in the complementary strand. For PI viruses L/T2 + C and L/T2 + T3, viral dsRNA genes were first converted to cDNAs by reverse transcription and PCR using previously described techniques (15) and then cloned into the pCRII vector (Invitrogen, San Diego, Calif.). Sequences of S4 gene cDNAs were determined by dideoxy-chain termination reactions using oligodeoxynucleotide primers, T7 DNA polymerase (U.S. Biochemical, Cleveland, Ohio), and [³⁵S]dATP. Mutations in L/T2 + C and L/T2 + T3 S4 gene cDNAs and sequences of nontranslated regions were confirmed by using reverse transcriptase to directly determine dsRNA sequences (6, 28).

Treatment of reovirus virions with chymotrypsin. Purified virions of reovirus at a concentration of 8×10^{12} particles per ml in virion storage buffer (150 mM NaCl, 10 mM MgCl₂, 10 mM Tris [pH 7.4]) were digested for defined intervals with 200 μ g of *N*- α -tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine α -chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) per ml at 10°C. Chymotrypsin digestion was stopped by adding 2 mM phenylmethylsulfonyl fluoride to the treatment mixtures and cooling at 0°C.

SDS-PAGE of reovirus structural proteins. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (23). Aliquots of chymotrypsin-treatment mixtures of virions were mixed 1:1 with 2 $\times$ sample buffer (250 mM Tris, 4% 2-mercaptoethanol, 2% SDS, 0.02% bromophenol blue) and incubated at 65°C for 5 min. Samples were loaded into wells of 5 to 15% polyacrylamide gradient gels and electrophoresed at 10-mA constant current for 18 h. Gels were stained with Coomassie blue R-250 (Sigma) and photographed.

Densitometric analysis of reovirus outer-capsid proteins. Photographs of Coomassie blue-stained gels were scanned by using the scanning program Ofoto, version 2.0 (Light Source Computer Images, San Rafael, Calif.). Scanned images were transferred to the digital image processing and analysis program Image 1.33f, version 1.25 (Research Services Branch, National Institute of Mental Health, Bethesda, Md.). For each time point of chymotrypsin digestion, mean densities were determined for bands corresponding to the δ cleavage product of the μ 1C protein, the $\sigma 2$ protein, and the $\sigma 3$ protein. Total densities of bands corresponding to δ and $\sigma 3$ were divided by the density of the band corresponding to $\sigma 2$ at each time point as a control for loading. Core protein $\sigma 2$ is not degraded during chymotrypsin treatment of virions to generate ISVPs (9, 12, 27, 36, 38).

RESULTS

Identification of viral genes that segregate with PI virus growth in the presence of ammonium chloride. Reoviruses isolated from persistently infected carrier cultures can grow in the presence of the disassembly inhibitor ammonium chloride (16, 41) (Table 1). To determine mechanisms by which mutations selected during persistent reovirus infection alter acid-dependent proteolytic disassembly of the viral outer capsid, we used reassortant genetics to identify viral genes associated with growth of PI viruses in the presence of ammonium chloride. Reassortant viruses isolated from independent crosses of wt T1L and three PI viruses, L/C, PI 2A1, and PI 3-1, were used for these experiments. L/C was isolated from a persistently infected culture established with T3D temperature-sensitive mutant *ts*C447 (1). PI 2A1 and PI 3-1 were isolated from cultures established with independent high-passage stocks of T3D (16). As shown in Fig. 1, each of these PI viruses produced approximately 50-fold-greater yields than T1L after 24 h of growth in L cells treated with 10 mM ammonium chloride. Reassortant viruses isolated from each cross were used to infect L cells maintained in the presence or absence of 10 mM ammonium chloride, and viral yields were determined by plaque assay after 24 h of viral growth. Yields in ammonium chloride-treated cells were divided by those in untreated cells to calculate an AC/L ratio for each reassortant virus (Tables 2 to 4).

In the case of the T1L $\times$ L/C reassortants, we found that the S1 gene segregated with viral growth in the presence of ammonium chloride. Reassortant viruses containing an S1 gene derived from L/C had AC/L ratios of >0.1 , whereas those with

FIG. 1. Growth of wt T1L and PI viruses L/C, PI 2A1, and PI 3-1 in the presence (A) and absence (B) of ammonium chloride (AC). Monolayers of L cells (5×10^5) were infected with either T1L or each of the PI viruses at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium was added with or without 10 mM ammonium chloride, and the cells were incubated at 37°C for the indicated intervals. Cells were frozen and thawed twice, and virus in cell lysates was titrated on L-cell monolayers by plaque assay. The results are presented as the mean viral titer for two independent experiments.

an S1 gene from T1L had AC/L ratios of <0.1 (Table 2). No other reovirus genes were associated with AC/L ratios of >0.1 , which suggests that mutations in the S1 gene determine the capacity of L/C to grow better than wt virus in the presence of

ammonium chloride. To confirm the association of the S1 gene with growth of T1L $\times$ L/C reassortants in ammonium chloride-treated cells, we analyzed the results by the nonparametric Mann-Whitney test. The results of this analysis demonstrated a significant association between growth of reassortant viruses in the presence of ammonium chloride and the S1 gene ($P = 0.037$). No other viral genes were significantly associated with growth of T1L $\times$ L/C reassortants in ammonium chloride-treated cells ($P > 0.05$).

In the case of the T1L $\times$ PI 2A1 and T1L $\times$ PI 3-1 reassortants, however, we found that the S4 gene segregated with viral growth in the presence of ammonium chloride. Reassortants from either cross containing an S4 gene from the PI virus parent had AC/L ratios of >0.015 , whereas those with an S4 gene from T1L had AC/L ratios of ≤ 0.015 (Tables 3 and 4). The association of the S4 gene with growth of T1L $\times$ PI 2A1 and T1L $\times$ PI 3-1 reassortant viruses in the presence of ammonium chloride was statistically significant in each case (T1L $\times$ PI 2A1 reassortants, $P = 0.007$; T1L $\times$ PI 3-1 reassortants, $P = 0.012$). No other viral genes were significantly associated with growth of reassortants from either cross in ammonium chloride-treated cells ($P > 0.05$).

As a control to exclude the possibility that certain combinations of T1L and T3D genes could confer viral growth in the presence of ammonium chloride, eight reassortant viruses isolated from crosses of T1L and T3D (11, 14, 40), the wt strain from which these PI viruses were derived (1, 16), were tested for growth in ammonium chloride-treated cells (data not shown). None of these reassortants grew significantly better than the parental strains in the presence of ammonium chloride. Therefore, mutations selected during persistent infection in either the S1 gene or the S4 gene are associated with growth of PI viruses in L cells treated with ammonium chloride.

PI virus S4 gene nucleotide sequences. In previous work, we found that mutations in PI virus S1 genes resulted in amino acid substitutions in the tail region of viral attachment protein $\sigma 1$ (42). To identify mutations in PI virus S4 genes, we determined the S4 gene nucleotide sequences of L/C, PI 2A1, and PI 3-1 and of four additional PI viruses, L/T2 + C, L/T2 + T3, PI 1A1, and PI 5-1. These studies were conducted to determine whether mutations selected during persistent infection in PI

TABLE 2. Growth of T1L $\times$ L/C reassortant viruses in the presence and absence of ammonium chloride

Virus strain	Origin of gene segment ^a										Yield in ^b :		AC/L ratio ^c	Rank ^d
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	AC-treated cells	Untreated cells		
Parental														
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	4.8×10^5	1.0×10^8	0.0046	
L/C	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	7.6×10^8	1.4×10^9	0.55	
Reassortant														
RK23	PI	wt	PI	PI	PI	PI	PI	PI	PI	PI	5.0×10^7	1.8×10^8	0.28	1
RK87	wt	PI	wt	PI	wt	wt	PI	PI	wt	wt	8.8×10^7	4.4×10^8	0.20	2
RK103	PI	PI	PI	PI	PI	PI	PI	PI	wt	wt	9.5×10^7	5.0×10^8	0.19	3
RK41	PI	PI	wt	wt	wt	PI	PI	PI	PI	wt	5.0×10^7	3.6×10^8	0.14	4
RK49	wt	PI	wt	wt	wt	PI	PI	wt	wt	PI	7.0×10^7	5.2×10^8	0.13	5
RK99	wt	PI	wt	wt	wt	PI	wt	PI	PI	wt	9.1×10^6	2.8×10^8	0.033	6
RK26	PI	PI	PI	PI	PI	PI	wt	PI	wt	PI	8.8×10^6	2.9×10^8	0.030	7
RK96	PI	PI	PI	PI	PI	PI	wt	PI	PI	PI	8.6×10^6	4.0×10^8	0.021	8

^a Parental origin of each gene segment: wt, gene segment derived from T1L; PI, gene segment derived from L/C.

^b L cells (5×10^5) were infected with virus strains at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium with or without 10 mM ammonium chloride (AC) was added, and the cells were incubated at 37°C for 24 h. Cells were frozen and thawed twice prior to being titrated on L-cell monolayers by plaque assay. The results are presented as mean viral yields for 30 experiments for T1L, 6 experiments for L/C, and 4 experiments for each of the T1L $\times$ L/C reassortant viruses.

^c The mean viral yield in ammonium chloride-treated cells was divided by that in untreated L cells to calculate an AC/L ratio for each virus strain.

^d Reassortant viruses are ranked from highest to lowest based on their AC/L ratios.

TABLE 3. Growth of T1L $\times$ PI 2A1 reassortant viruses in the presence and absence of ammonium chloride

Virus strain	Origin of gene segment ^a										Yield in ^b :		AC/L ratio ^c	Rank ^d
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	AC-treated cells	Untreated cells		
Parental strains														
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	4.8×10^5	1.0×10^8	0.0046	
PI 2A1	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	3.9×10^7	2.3×10^8	0.17	
Reassortant														
JW106	PI	PI	PI	PI	wt	PI	PI	PI	wt	PI	1.0×10^8	1.9×10^8	0.53	1
JW35	PI	PI	PI	wt	wt	wt	PI	wt	wt	PI	7.5×10^7	1.9×10^8	0.39	2
JW24	PI	PI	PI	PI	PI	wt	PI	PI	wt	PI	1.4×10^6	5.9×10^6	0.24	3
JW34	wt	wt	wt	wt	wt	PI	wt	wt	PI	PI	1.0×10^6	1.3×10^7	0.077	4
JW22	PI	PI	PI	PI	wt	PI	wt	wt	PI	PI	4.8×10^6	7.0×10^7	0.069	5
JW1	PI	PI	PI	wt	wt	PI	wt	PI	wt	PI	1.5×10^6	4.3×10^7	0.035	6
JW222	PI	PI	wt	PI	PI	PI	wt	PI	PI	PI	2.9×10^6	8.3×10^7	0.035	7
JW33	PI	PI	PI	wt	PI	wt	wt	PI	PI	PI	1.7×10^5	5.4×10^6	0.031	8
JW36	PI	PI	PI	PI	PI	PI	PI	PI	wt	PI	4.5×10^5	2.7×10^7	0.017	9
JW52	wt	wt	wt	wt	wt	wt	wt	PI	PI	wt	4.7×10^6	3.2×10^8	0.015	10
JW101	wt	PI	wt	wt	wt	wt	wt	PI	PI	wt	4.1×10^5	4.8×10^7	0.0085	11
JW260	PI	PI	wt	PI	PI	PI	wt	PI	PI	wt	2.3×10^5	3.6×10^7	0.0064	12
JW12	PI	PI	PI	wt	wt	wt	wt	wt	wt	wt	5.0×10^5	1.0×10^8	0.005	13

^a Parental origin of each gene segment: wt, gene segment derived from T1L; PI, gene segment derived from PI 2A1.

^b L cells (5×10^5) were infected with virus strains at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium with or without 10 mM ammonium chloride (AC) was added, and the cells were incubated at 37°C for 24 h. Cells were frozen and thawed twice prior to being titrated on L-cell monolayers by plaque assay. The results are presented as mean viral yields for 30 experiments for T1L, 11 experiments for PI 2A1, and 4 experiments for each of the T1L $\times$ PI 2A1 reassortant viruses.

^c The mean viral yield in ammonium chloride-treated cells was divided by that in untreated L cells to calculate an AC/L ratio for each virus strain.

^d Reassortant viruses are ranked from highest to lowest based on their AC/L ratios.

virus S4 genes resulted in amino acid substitutions in specific regions of viral outer-capsid protein $\sigma 3$. L/T2 + C and L/T2 + T3 were isolated from persistently infected L-cell cultures established with coinfections of T2J and either tsC447 (L/T2 + C) or T3D (L/T2 + T3) (2); PI 1A1 and PI 5-1 were isolated from persistently infected cultures established with independent high-passage stocks of T3D (16). Each of the PI viruses

studied produced approximately 50-fold-greater yields than wt virus when grown in ammonium chloride-treated cells (Table 1). S4 gene dsRNA was purified from PI virus virions, and full-length sequences of the coding strand were determined for each strain (Table 5). Few mutations were found in the S4 sequences of these viruses (four or fewer mutations per S4 gene), and with a single exception, each mutation resulted in a

TABLE 4. Growth of T1L $\times$ PI 3-1 reassortant viruses in the presence and absence of ammonium chloride

Virus strain	Origin of gene segment ^a										Yield in ^b :		AC/L ratio ^c	Rank ^d
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	AC-treated cells	Untreated cells		
Parental strains														
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	4.8×10^5	1.0×10^8	0.0046	
PI 3-1	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	6.5×10^6	7.9×10^7	0.081	
Reassortant														
LD10	PI	PI	wt	wt	wt	PI	PI	PI	wt	PI	1.4×10^7	6.7×10^7	0.20	1
LD134	wt	wt	wt	wt	wt	PI	PI	wt	wt	PI	2.3×10^6	4.1×10^7	0.056	2
LD15	PI	PI	PI	PI	PI	PI	wt	PI	PI	PI	1.3×10^7	2.7×10^8	0.049	3
LD125	PI	PI	wt	PI	PI	PI	wt	PI	PI	PI	2.4×10^6	7.6×10^7	0.032	4
LD144	wt	wt	wt	PI	PI	wt	wt	PI	PI	PI	8.3×10^5	4.2×10^7	0.020	5
LD4	wt	PI	wt	wt	wt	PI	wt	wt	wt	wt	9.3×10^4	8.7×10^6	0.011	6
LD18	PI	PI	PI	PI	PI	PI	wt	PI	PI	wt	4.1×10^5	7.6×10^7	0.0053	7
LD145	wt	wt	wt	wt	wt	wt	PI	wt	wt	wt	7.7×10^5	1.8×10^8	0.0042	8
LD127	wt	wt	wt	wt	wt	wt	wt	wt	PI	wt	3.9×10^4	6.7×10^7	0.00058	9
LD110	PI	PI	wt	PI	PI	PI	PI	PI	wt	wt	3.9×10^4	1.1×10^8	0.00035	10

^a Parental origin of each gene segment: wt, gene segment derived from T1L; PI, gene segment derived from PI 3-1.

^b L cells (5×10^5) were infected with virus strains at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium with or without 10 mM ammonium chloride (AC) was added, and the cells were incubated at 37°C for 24 h. Cells were frozen and thawed twice prior to being titrated on L-cell monolayers by plaque assay. The results are presented as mean viral yields for 30 experiments for T1L, 12 experiments for PI 3-1, and 2 to 4 experiments for each of the T1L $\times$ PI 3-1 reassortant viruses.

^c The mean viral yield in ammonium chloride-treated cells was divided by that in untreated L cells to calculate an AC/L ratio for each virus strain.

^d Reassortant viruses are ranked from highest to lowest based on their AC/L ratios.

TABLE 5. Mutations in S4 gene nucleotide sequences of prototype PI viruses and corresponding mutations in deduced amino acid sequences of their $\sigma 3$ proteins

PI virus	Location of mutation ^a		GenBank accession no.
	S4 gene	$\sigma 3$ protein	
L/C	109, A→G	26, Y→C	U63307
	420, G→A	130, E→K	
	1092, U→C	354, Y→H	
PI 2A1	420, G→A	130, E→K	U63305
	1092, U→C	354, Y→H	
	1157, U→C		
PI 3-1	1092, U→C	354, Y→H	U63304
	358, U→G	109, M→R	
	684, U→G	218, W→G	
L/T2 + C	1092, U→C	354, Y→H	U63309
	290, G→U	86, Q→H	
	466, U→C	145, I→T	
L/T2 + T3	726, C→U	232, P→S	U63308
	1092, U→C	354, Y→H	
	711, A→C	227, E→A	
PI 1A1	1092, U→C	354, Y→H	U63306
PI 5-1	1092, U→C	354, Y→H	U63303

^a Mutations in the S4 gene refer to nucleotide position; mutations in the $\sigma 3$ protein refer to amino acid position.

substitution in the deduced amino acid sequence of $\sigma 3$ protein. A common tyrosine-to-histidine substitution was found at amino acid 354 in the $\sigma 3$ amino acid sequences of L/C, PI 2A1, PI 3-1, L/T2 + C, L/T2 + T3, and PI 5-1. L/C and PI 2A1 also contained a common glutamate-to-lysine substitution at amino acid 130. Neither of these substitutions was observed in the deduced amino acid sequences of $\sigma 3$ protein from two stocks of T3D maintained in our laboratory (data not shown). PI 1A1 contained a unique glutamate-to-alanine substitution at amino acid 227. The only mutation in the nontranslated regions of these PI virus S4 genes was found at nucleotide 1157 in PI 2A1 (Table 5).

Cleavage of wt and PI virus outer-capsid proteins during virion-to-ISVP conversion in vitro. The reovirus $\sigma 3$ and $\mu 1C$ proteins are major components of the viral outer capsid (17, 37), and both proteins are cleaved during conversion of virions to ISVPs (12, 36, 38). We reasoned that PI viruses, which are capable of growth in the presence of disassembly inhibitor ammonium chloride, might undergo virion-to-ISVP processing more efficiently than wt virus. To determine whether mutations in PI viruses affect cleavage susceptibility of viral outer-capsid proteins during conversion of virions to ISVPs, purified virions of wt parental virus T3D and PI viruses L/C, PI 2A1, and PI 3-1 were treated with chymotrypsin in vitro for various intervals and analyzed by SDS-PAGE (Fig. 2). Marked differences were noted in the kinetics of cleavage of wt and PI virus outer-capsid proteins during chymotrypsin treatment of these viruses. In the case of wt T3D, a protein band corresponding to $\sigma 3$ was clearly visible after 30 min of chymotrypsin treatment. However, in the case of the PI viruses, the $\sigma 3$ band was lost after 15 min of chymotrypsin treatment. Similarly, a band corresponding to the δ cleavage fragment of the $\mu 1C$ protein was noted after 15 min of chymotrypsin treatment of T3D, whereas δ bands were clearly visible at times preceding 15 min of chymotrypsin treatment of the PI viruses. These results were quantitated by densitometric analysis of the $\sigma 3$ and δ bands during chymotrypsin treatment of the wt and PI viruses (Fig. 3). The findings show that the $\sigma 3$ proteins of the PI viruses were degraded faster than the $\sigma 3$ protein of wt T3D. Concordantly, appearance of the PI virus δ fragments occurred

FIG. 2. Electrophoretic analysis of viral structural proteins of wt and PI virus virions during treatment with chymotrypsin to generate ISVPs. Purified virions of wt T3D and PI viruses L/C, PI 2A1, and PI 3-1 at a concentration of 8×10^{12} particles per ml were treated with chymotrypsin at 10°C for the times shown. Equal volumes of samples (75 μ l) were loaded into wells of 5 to 15% polyacrylamide gradient gels. After electrophoresis, gels were stained with Coomassie blue. Times of chymotrypsin treatment are shown above the gels. Viral proteins are labeled, and positions of molecular weight markers in kilodaltons appear in the lanes labeled M.

FIG. 3. Densitometry of wt and PI virus $\sigma 3$ protein (A) and δ cleavage fragment of $\mu 1C$ protein (B) during chymotrypsin treatment of virions to generate ISVPs. Band intensities were first standardized to those of bands corresponding to core protein $\sigma 2$ at each time point as a control for loading. Intensities of $\sigma 3$ protein and δ cleavage fragment were then standardized to 0 and 60 min, respectively, which represent times of maximum band intensity for each protein in these experiments.

FIG. 4. Electrophoretic analysis of viral structural proteins of wt and PI virus virions in the absence of chymotrypsin treatment. Purified virions of wt T3D and PI viruses PI 2A1, PI 3-1, and L/C at a concentration of 8×10^{12} particles per ml were incubated in chymotrypsin treatment buffer at 10°C for the times shown. Equal volumes of samples ($75 \mu\text{l}$) were loaded into wells of 5 to 15% polyacrylamide gradient gels. After electrophoresis, gels were stained with Coomassie blue. Times of incubation are shown above the gel. Viral proteins are labeled, and positions of molecular weight markers in kilodaltons appear in the lane labeled M.

earlier than appearance of the δ fragment of T3D. Similar findings were made in two additional experiments (data not shown). These results indicate that viral mutations selected during persistent reovirus infection alter kinetics of σ_3 and $\mu_1\text{C}$ proteolysis during chymotrypsin treatment of virions in vitro to generate ISVPs as end products.

In these experiments, faintly visible δ bands were detected at the initiation of the chymotrypsin treatment course for each of the PI viruses studied. To determine whether PI virus σ_3 and $\mu_1\text{C}$ proteins are stable in the absence of chymotrypsin treatment, purified virions of wt T3D and PI viruses L/C, PI 2A1, and PI 3-1 were incubated in chymotrypsin treatment buffer for various intervals and analyzed by SDS-PAGE (Fig. 4). The results show that the σ_3 and $\mu_1\text{C}$ bands of wt and PI viruses are stable for at least 3 h of incubation. These findings also were corroborated by densitometric analysis of the σ_3 and $\mu_1\text{C}$ bands during the incubation time course (data not shown). Therefore, outer-capsid proteins of wt and PI viruses do not differ in stability in the absence of protease treatment.

DISCUSSION

Mutations in either the S1 gene or the S4 gene affect acid-dependent proteolytic disassembly of reovirus virions. In this report, we show that mutations in either the S1 or S4 gene selected during persistent infection determine the capacity of

PI reoviruses to grow in cells treated with ammonium chloride. Ammonium chloride is a weak base that increases pH in endosomes (25) and lysosomes (32) and blocks proteolytic processing of reovirus virions to ISVPs (16, 38). The σ_1 -encoding S1 gene segregates with growth of T1L $\times$ L/C reassortants in ammonium chloride-treated cells, whereas the σ_3 -encoding S4 gene segregates with growth of both T1L $\times$ PI 2A1 and T1L $\times$ PI 3-1 reassortants in cells treated with ammonium chloride. These findings suggest that acid-dependent disassembly events involve at least two viral outer-capsid proteins during conversion of virions to ISVPs.

Our previous studies of PI virus σ_1 proteins indicate that mutations selected in σ_1 during persistent infection decrease stability of σ_1 oligomers at increased pH (42). The σ_1 protein undergoes a conformational change during in vitro treatment of virions with chymotrypsin to generate ISVPs (17, 18, 27), and it is postulated that this conformational change in σ_1 during virion-to-ISVP conversion in reovirus-infected cells is acid dependent (42). Conformational changes in σ_1 protein might be required for σ_1 to disengage from cellular receptors following receptor-mediated uptake into cellular endosomes or to facilitate interactions with other outer-capsid proteins, such as σ_3 , $\mu_1/\mu_1\text{C}$, or λ_2 , necessary for viral disassembly. Therefore, mutations in σ_1 protein selected during persistent infection might facilitate PI virus growth in the presence of ammonium chloride by allowing σ_1 to perform conformationally dependent functions at a more basic pH.

The σ_3 protein is degraded and lost from virions during conversion of virions to ISVPs (9, 12, 36, 38). During viral entry, proteolysis of σ_3 is acid dependent (38); however, it is not known whether acidic pH is required for optimal activity of cellular proteases or whether it facilitates a conformational change in σ_3 that results in increased susceptibility of the protein to cleavage. The observation that PI virus σ_3 proteins are cleaved with faster kinetics than wt virus σ_3 after in vitro treatment with chymotrypsin suggests that protease sensitivity of PI virus σ_3 proteins is increased in comparison to wt σ_3 . Thus, it appears that mutations in σ_3 protein selected during persistent infection alter its intrinsic susceptibility to cleavage. Of note, this effect appears to be a property of PI viruses in which mutations that confer growth in the presence of ammonium chloride map to either the S1 gene (L/C) or the S4 gene (PI 2A1 or PI 3-1). This finding suggests that proteolytic cleavage of σ_3 is affected by both inter- and intramolecular interactions in the viral outer capsid.

FIG. 5. Locations of mutations in deduced σ_3 amino acid sequences of PI reoviruses. (A) Functional domains of σ_3 protein (26, 34). A site cleaved by staphylococcal V8 protease is indicated by an arrow (34). Amino acid positions in σ_3 sequence are shown. (B) Mutations in σ_3 proteins of seven PI viruses. Viral genes that determine growth in cells treated with ammonium chloride (AC) are indicated for PI virus strains L/C, PI 2A1, and PI 3-1. —, genetic analysis not performed.

The PI viruses used in this study were selected during persistent infection by growth in mutant cells that do not fully support virion-to-ISVP processing (16). Therefore, it was anticipated that mutations conferring growth of PI viruses in mutant cells (22, 42) and in cells treated with ammonium chloride (this report) would map to genes encoding outer-capsid proteins. However, viral genes that determine these two phenotypes are not identical for all strains. In the cases of L/C and PI 2A1, both growth in mutant cells and growth in ammonium chloride-treated cells map to the same viral genes (S1 in the case of L/C and S4 in the case of PI 2A1). In the case of PI 3-1, however, growth in mutant cells maps to the S1 gene, whereas growth in ammonium chloride-treated cells maps to the S4 gene. These findings indicate that either different mechanisms or a single mechanism facilitated by different viral genes can be used by mutant viruses to bypass blocks to viral entry present in mutant cells and in cells treated with ammonium chloride. Moreover, the observation that mutations in $\sigma 1$, $\sigma 3$, or both proteins can confer PI virus growth in mutant cells or in ammonium chloride-treated cells suggests that $\sigma 1$ and $\sigma 3$ interact to facilitate disassembly of reovirus virions.

Mutations in PI virus $\sigma 3$ proteins. As for the nucleotide sequences of PI virus S1 genes that were determined in a previous study (42), few mutations were observed in the S4 genes of the PI viruses reported here. This finding was unexpected given the prolonged period for which these viruses were maintained in culture. Three regions in $\sigma 3$ protein appear to be targets for mutations in PI viruses: amino acids 86 to 145, 218 to 232, and 354 (Fig. 5). Of these, only the mutation at amino acid 354 can be conclusively linked to growth in the presence of ammonium chloride. A tyrosine-to-histidine mutation at amino acid 354 is the only change in the deduced amino acid sequence of PI 3-1 $\sigma 3$ protein, and growth of T1L $\times$ PI 3-1 reassortant viruses in ammonium chloride-treated cells segregates exclusively with the $\sigma 3$ -encoding S4 gene.

Current understanding of $\sigma 3$ structure and function indicates that an amino-terminal domain, which includes a zinc finger motif at residues 51 to 73, binds zinc (34) and a carboxy-terminal domain, which includes basic amino acid motifs at residues 234 to 240 and 291 to 297, binds dsRNA (26). A region of $\sigma 3$ adjacent to amino acid 220 is sensitive to a variety of proteases (26, 34), and this region of the protein is postulated to be cleaved by endocytic proteases during viral entry (35). We hypothesize that the primary effect of $\sigma 3$ mutations in PI reoviruses is to enhance susceptibility of $\sigma 3$ to proteolysis. This enhancement might be accomplished by altering the conformation of the $\sigma 3$ cleavage site or by altering interactions between $\sigma 3$ and another outer-capsid protein such that the $\sigma 3$ cleavage site is rendered more susceptible to proteolytic attack. In support of this idea, it has been shown that interactions between $\sigma 3$ and $\mu 1$ result in a conformational change in $\sigma 3$ that increases its susceptibility to cleavage (35).

Although growth of L/C in ammonium chloride-treated cells maps to the S1 gene and growth of PI 2A1 and PI 3-1 in ammonium chloride-treated cells maps to the S4 gene, the deduced amino acid sequences of the L/C, PI 2A1, and PI 3-1 $\sigma 3$ proteins contain the same mutation at amino acid 354. However, the $\sigma 3$ protein of L/C contains an additional mutation, tyrosine to cysteine at amino acid 26, that is not found in the corresponding proteins of PI 2A1 and PI 3-1. It is possible that this mutation serves to suppress the effect of the mutation at amino acid 354 on determining viral growth in the presence of ammonium chloride.

PI viruses and studies of viral disassembly. Mutant viruses selected during persistent infections of cultured cells have

proven to be useful tools for studies of viral disassembly. Our studies of PI reoviruses argue that susceptibility of $\sigma 3$ to protease cleavage is an important determinant of the capacity of reovirus to maintain persistent infections of cultured cells. In addition, our findings show that acid-dependent disassembly events involving the $\sigma 1$ protein also influence the maintenance of persistent reovirus infections. Together, these results suggest that the $\sigma 1$ and $\sigma 3$ proteins represent important targets for mutations that promote viral adaptation to growth in mutant cells in the context of persistently infected cultures. Ongoing investigations of the PI viruses reported here, in addition to those reported in previous studies (41), should clarify the roles of $\sigma 1$ conformational changes and $\sigma 3$ cleavage susceptibility in reovirus disassembly and provide new insights into mechanisms by which nonenveloped viruses enter their host cells.

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