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Persistent Reovirus Infections of L Cells Select Mutations in Viral Attachment Protein σ1 That Alter Oligomer Stability

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During maintenance of L-cell cultures persistently infected with reovirus, mutations are selected in viruses and cells. Cells cured of persistent infection support growth of viruses isolated from persistently infected cultures (PI viruses) significantly better than that of wild-type (wt) viruses. In a previous study, the capacity of PI virus strain L/C to grow better than wt strain type 1 Lang (T1L) in cured cells was mapped genetically to the S1 gene (R. S. Kauffman, R. Ahmed, and B. N. Fields, Virology 131:79–87, 1983), which encodes viral attachment protein σ1. To investigate mechanisms by which mutations in S1 confer growth of PI viruses in cured cells, we determined the S1 gene nucleotide sequences of L/C virus and six additional PI viruses isolated from independent persistently infected L-cell cultures. The S1 sequences of these viruses contained from one to three mutations, and with the exception of PI 2A1, mutations in each S1 gene resulted in changes in the deduced amino acid sequence of σ1 protein. Using electrophoresis conditions that favor migration of σ1 oligomers, we found that σ1 proteins of L/C, PI 1A1, PI 3-1, and PI 5-1 migrated as monomers, whereas σ1 proteins of wt reovirus and PI 2A1 migrated as oligomers. These findings suggest that mutations in σ1 protein affecting stability of σ1 oligomers are important for the capacity of PI viruses to infect mutant cells selected during persistent infection. Since no mutation was found in the deduced amino acid sequence of PI 2A1 σ1 protein, we used T1L × PI 2A1 reassortant viruses to identify viral genes associated with the capacity of this PI virus to grow better than wt in cured cells. The capacity of PI 2A1 to grow better than T1L in cured cells was mapped to the S4 gene, which encodes outer-capsid protein σ3. This finding suggests that in some cases, mutations in σ3 protein in the absence of σ1 mutations confer growth of PI viruses in mutant cells. To confirm the importance of the S1 gene in PI virus growth in cured cells, we used T1L × PI 3-1 reassortant viruses to genetically map the capacity of this PI virus to grow better than wt in cured cells. In contrast to our results using PI 2A1, we found that growth of PI 3-1 in cured cells was determined by the σ1-encoding S1 gene. Given that the σ1 and σ3 proteins play important roles in reovirus disassembly, findings made in this study suggest that stability of the viral outer capsid is an important determinant of the capacity of reoviruses to adapt to host cells during persistent infection.

Mammalian reoviruses are useful models for studies of mechanisms that promote persistent viral infections of cultured cells. Maintenance of persistent reovirus infections of murine L929 (L) cells (1, 17) and murine erythroleukemia cells (55) is associated with selection of mutations in both viruses and cells. When such cultures are cured of persistent infection by treatment with antireovirus antibodies, the resulting cells are more permissive for viruses isolated from the persistently infected cultures (PI viruses) than wild-type (wt) viruses (1, 17, 55). In one study, reassortant viruses containing an S1 gene derived from PI virus L/C, which was isolated from a persistently infected L-cell culture (1), grew to high titer in cured cells, suggesting that mutations in S1 are important for maintenance of persistent infection (27). The S1 gene encodes two proteins, σ1 and σ1s, in overlapping reading frames (21, 25, 43). The σ1 protein is the viral attachment protein (29, 52), hemagglutinin (41, 54), and determinant of the type-specific neutralizing immune response (53); σ1s is a nonstructural protein of unknown function. The S1 gene product that determines differences in the capacity of wt and PI viruses to grow in cured cells has not been identified.

Studies of L-cell (17) and murine erythroleukemia cell (55) cultures persistently infected with reovirus suggest that cellular mutations selected during maintenance of persistent infection result in a block to viral disassembly. Although cells cured of persistent infection do not support efficient growth of wt viruses after infection by virions, they do after infection by infectious subvirion particles (ISVPs) (17, 55), which are intermediates in reovirus disassembly that can be generated in vitro by treatment of virions with chymotrypsin (10, 26, 44). During viral entry into cells, ISVPs are generated by acid-dependent proteolysis of viral outer-capsid proteins σ3 and μ1/μ1C in an endocytic compartment (48). Therefore, the capacity of wt viruses to grow in cured cells after infection by ISVPs indicates that a mutation in these cells likely affects virion-to-ISVP processing. Given that PI viruses are similar to ISVPs in that they can infect cured cells, it is likely that viral mutations selected during persistent infection affect steps in reovirus replication leading to generation of ISVPs. Concordantly, PI viruses grow much better than wt viruses in L cells treated with ammonium chloride (17, 55), a weak base that blocks proteolytic processing of the viral outer capsid (48). However, mechanisms by

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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[c] stock	Growth in cured cells (LX/L ratio)[d]
L/C	281	L/C	*ts*C447	0.46
L/T2 + C	304	L/T2 + C	wt T2J + hp *ts*C447	3.6
L/T2 + T3	294	L/T2 + T3	wt T2J + hp T3D	1.6
PI 1A1	151	L/T3D-1A	T3D-1	0.14
PI 2A1	151	L/T3D-2A	T3D-2	3.1
PI 3-1	178	L/T3D-3	T3D-3	0.59
PI 5-1	89	L/T3D-5	T3D-5	0.15

[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 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 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 (17).

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

[c] hp, high-passage.

[d] Cured LX cells or wt L cells (5×10^5) were infected with virus strains at an MOI of 0.1 PFU per cell and incubated at 37°C for 72 h. Cells were frozen and thawed twice and titrated on L-cell monolayers by plaque assay. Experiments were performed in quadruplicate, and the mean viral yield in LX cells was divided by that in L cells to calculate an LX/L ratio for each virus strain. The LX/L ratio for wt strain T3D was 0.034. Data for T3D, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 were reported previously (17).

which mutations in PI viruses promote viral disassembly in mutant cells are not known.

To better understand how mutations in the viral S1 gene favor maintenance of persistent reovirus infection, we determined the S1 gene nucleotide sequences of seven PI viruses isolated from independent persistently infected L-cell cultures. We conducted experiments to determine whether mutations in σ1 protein alter stability of σ1 oligomers, and we used reassortant viruses to determine whether viral genes in addition to S1 are associated with the capacity of PI viruses to grow better than wt viruses in cured cells. The results provide insight into mechanisms used by reovirus to maintain persistent infections of cultured cells.

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 μg of streptomycin, and 250 ng of amphotericin B per ml (Irvine). LX-2 (LX) cells, which were cured of persistent reovirus infection by treatment with antireovirus antibodies (17), were cultivated under identical conditions except that the medium was supplemented to contain 10% fetal bovine serum. Reovirus strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) are laboratory stocks. PI viruses L/C, L/T2 + C, L/T2 + T3, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 were isolated previously from independent persistently infected L-cell cultures (2, 3, 17) (Table 1). Purified virion preparations were made by using second-passage L-cell lysate stocks of twice-plaque-purified reovirus and cesium chloride gradient centrifugation as previously described (23). Following cesium chloride gradient centrifugation, virions were dialyzed against virion storage buffer lacking divalent cations (150 mM NaCl, 10 mM Tris [pH 7.5]).

Sequencing PI virus S1 genes. For PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1, genomic S1 gene double-stranded RNA (dsRNA) was purified from virions and sequenced by using dideoxy sequencing reactions with deoxyoligonucleotide primers, reverse transcriptase (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.), and [^{35}S]dATP (1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) as previously described (9, 37). Full-length sequences of coding and complementary strands were determined for each S1 gene. For PI viruses L/T2 + C and L/T2 + T3, viral dsRNA genes were first converted to cDNAs by reverse transcription and PCR by using previously described techniques (14) and then cloned into the pCRII vector (Invitrogen, San Diego, Calif.). Sequences of S1 gene cDNAs were determined by dideoxy-chain termination reactions using [^{35}S]dATP, oligodeoxynucleotide primers, and T7 DNA polymerase (U.S. Biochemical, Cleveland, Ohio). Mutations in L/T2 + C and L/T2 + T3 S1 gene cDNAs and sequences of nontranslated regions were confirmed by using reverse transcriptase to directly determine dsRNA sequences (9, 37).

SDS-PAGE of reovirus structural proteins. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (38). In preparation for electrophoresis, 8×10^{11} purified virions were mixed 1:1 with 2× sample buffer (125 mM Tris, 10% β-mercaptoethanol, 4% SDS, 0.02% bromophenol blue) and incubated at 65°C for 5 min. Sample buffer pH was varied from 6.0 to 10.5, and $CaCl_2$ concentration was varied from 0 to 15 mM. Samples were loaded into wells of 5 to 15% gradient polyacrylamide gels and electrophoresed at 15-mA constant current at 4°C for 14 h. Gels were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.) and dried between cellophane.

Binding of wt and PI viruses to L cells. Virus binding to L cells was analyzed by fluorescence-activated flow cytometry as previously described (42), with slight modifications. Purified virions (4×10^{10} particles) were adsorbed to 10^6 cells, and then a 1:20 dilution of rabbit antireovirus antiserum was added. The secondary antibody used was a 1:50 dilution of fluorescein isothiocyanate-conjugated goat $F(ab')_2$ directed against rabbit immunoglobulin G (Sigma). Fluorescence intensity was determined with a FACS IV flow cytometer (Becton Dickinson Co., San Jose, Calif.). Cells considered positive for virus binding were those demonstrating fluorescence intensity exceeding that displayed by cells treated with primary and secondary antibodies alone.

HA by wt and PI viruses. Purified virions were serially diluted twofold from 2.0×10^{11} to 1.0×10^8 particles in 0.05 ml of cold phosphate-buffered saline (PBS) into 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.). Citrate-preserved calf bovine erythrocytes (Colorado Serum Company, Denver, Colo.) were washed three times in cold PBS and suspended at a concentration of 1% (vol/vol). Erythrocytes (0.05 ml) were added to wells containing virus and incubated at 4°C for 2 h. The smallest number of viral particles sufficient to produce hemagglutination (HA) was designated to equal 1 HA unit. The HA titer was defined as the number of HA units per 2×10^{11} particles.

Growth of reovirus in wt L cells and cured LX cells. Monolayers of L cells and LX cells (5×10^5 cells per well) were infected with virus strains at a multiplicity of infection (MOI) of 2 PFU in 24-well plates (Costar). After a 1-h adsorption period at 4°C, the inoculum was removed, 1.0 ml of fresh medium was added, and the cells were incubated at 37°C for defined intervals. Cells were frozen and thawed twice prior to being titrated on L-cell monolayers by plaque assay (50).

Isolation and characterization of T1L × PI 2A1 and T1L × PI 3-1 reassortant viruses. Reassortant viruses were isolated as previously described (45). L-cell monolayers were coinfected with either T1L and PI 2A1 (JW series) or T1L and PI 3-1 (LD series) in a ratio of 1:1 at 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 in L cells. Genotypes of reassortant viruses were determined by SDS-PAGE of viral dsRNA purified from second-passage lysate stocks (P2 stocks). To purify viral dsRNA, aliquots of 250 μl of each P2 stock were combined with 750 μl of Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) in 1.5-ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, Pa.) and incubated at 25°C for 5 min. Following incubation, 200 μl of chloroform was added to each tube, and the samples were incubated at 25°C for 15 min and centrifuged at 13,600 × *g* at 4°C for 15 min. The aqueous phase was removed and combined with 500 μl of isopropanol, incubated at 25°C for 10 min, and centrifuged at 13,600 × *g* at 4°C for 15 min. Supernatants were removed, and the resultant pellets were washed in 1 ml of 75% ethanol and centrifuged. Supernatants were removed, and the pellets were lyophilized. Pellets were solubilized in 1× sample buffer and electrophoresed in 10% polyacrylamide gels at 35-mA constant current for 14 h. After electrophoresis, gels were stained with ethidium bromide (Fisher Scientific), and gene segments were visualized by UV irradiation. Gene segment mobilities of reassortant viruses were compared with those of parental strains to determine the parental origin of each gene segment.

RESULTS

PI virus S1 gene nucleotide sequences. To identify mutations in the S1 gene selected during persistent reovirus infections of L cells, we determined the S1 gene nucleotide sequences of the seven PI viruses shown in Table 1. Each of these viruses was isolated from an independent persistently infected culture after a minimum of 89 days in culture, and each produces greater yields than wt virus when grown in cured LX cells (Table 1). S1 gene dsRNA was purified from each PI virus strain, and full-length sequences of both coding and complementary strands were determined (Table 2). The S1 genes of PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 were derived from T3D. These PI viruses were isolated from carrier cultures established using high-passage stocks of either T3D temperature-sensitive mutant *ts*C447 (L/C) (3) or high-passage stocks of T3D (PI 1A1,

TABLE 2. Mutations in S1 gene nucleotide sequences of prototype PI viruses and corresponding mutations in deduced amino acid sequences of their σ1 and σ1s proteins

PI virus strain	Location of mutation[a]			GenBank accession no.
	S1 gene	σ1 protein	σ1s protein	
L/C	165, G→C	51, Q→H	32, R→T	U53413
	743, C→U	244, S→L	—	
L/T2 + C	41, A→G	10, R→G	—	U53415
	Δ590–613	Δ193–200	—	
L/T2 + T3	86, A→G	25, K→E	—	U53414
	Δ590–613	Δ193–200	—	
	725, A→C	238, I→L	—	
PI 1A1	284, U→G	91, L→R	72, S→A	U53412
PI 2A1	378, U→C	—	103, I→T	U53411
PI 3-1	378, U→G	122, D→E	103, I→S	U53410
	383, A→G	124, D→G	105, T→A	
PI 5-1	383, A→G	124, D→G	105, T→A	U53409

[a] Mutations in the S1 gene refer to nucleotide position; mutations in the σ1 and σ1s proteins refer to amino acid position. Δ, deletion; —, no mutation.

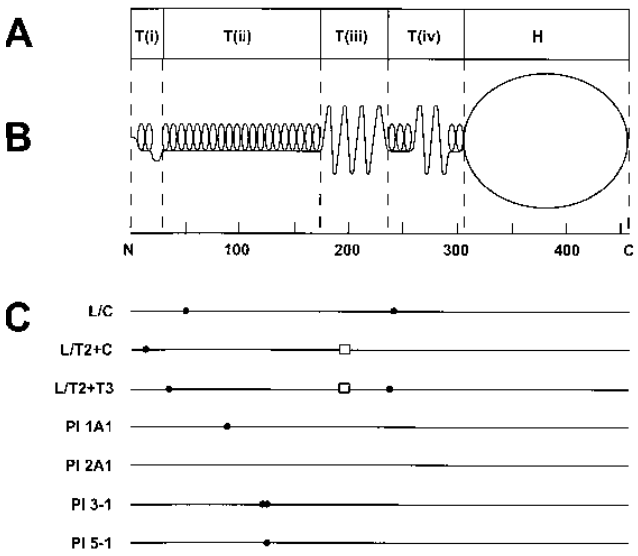

FIG. 1. Structural model of the reovirus σ1 protein and locations of mutations in the deduced σ1 amino acid sequences of seven PI viruses. (A) Morphologic regions of σ1 (tail [T] and head [H]) defined by analysis of electron microscopic images of purified σ1 (22). (B) Model of σ1 structure based on analysis of deduced σ1 amino acid sequences of prototype strains of the three reovirus serotypes (37). The fibrous tail is proposed to be constructed from a tandem arrangement of α-helix and β-sheet; the head is predicted to assume a more complex, globular structure. Regions of α-helix and β-sheet are indicated in the σ1 tail. Amino acid positions in T3D σ1 sequence are shown. (C) Mutations in the σ1 protein of the seven PI viruses. ●, site of point mutation; □, site of deletion.

PI 2A1, PI 3-1, and PI 5-1) (17). The S1 genes of PI viruses L/T2 + C and L/T2 + T3, however, were derived from T2J. L/T2 + C and L/T2 + T3 were isolated from persistently infected cultures established by using coinfections of wt T2J and high-passage stocks of either *ts*C447 (L/T2 + C) or T3D (L/T2 + T3) (2).

Few mutations were found in the S1 gene nucleotide sequences of the PI viruses studied (≤3 mutations per S1 gene). With the exception of the PI 2A1 S1 sequence, each of the S1 mutations predicts substitutions in the deduced amino acid sequence of σ1 protein. PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 also contained mutations in the deduced sequences of σ1s protein; however, no mutations were found in the deduced σ1s sequences of L/T2 + C and L/T2 + T3. Additionally, no mutations were observed in the nontranslated regions of the PI virus S1 genes. Migration of the S1 gene segments of L/T2 + C and L/T2 + T3 in SDS-polyacrylamide gels was previously shown to be faster than that of either the T2J or T3D S1 gene (2), which suggested that nucleotide sequences were deleted from the S1 genes of these viruses. We found that 24 nucleotides were deleted from the L/T2 + C and L/T2 + T3 S1 genes (nucleotides 590 to 613), resulting in deletion of eight amino acids in the deduced amino acid sequences of their σ1 proteins (residues 193 to 200). No deletions were observed in the S1 genes of PI viruses derived from T3D.

Mutations in PI virus σ1 protein affect the stability of σ1 oligomers. Reovirus attachment protein σ1 is a fibrous protein with a head-and-tail morphology (6, 22, 23). Mutations in σ1 proteins of L/C, L/T2 + C, L/T2 + T3, PI 1A1, PI 3-1, and PI 5-1 are located in the σ1 tail (Fig. 1). The amino terminus of the two mutations in L/C and each mutation in PI 1A1, PI 3-1, and PI 5-1 occur within a region of σ1 sequence previously shown to be important for the stability of σ1 oligomers (31). An oligomeric form of σ1 protein of wt T3D can be detected in SDS-polyacrylamide gels by increasing the pH of the sample buffer (8). To determine whether mutations in the σ1 proteins of L/C, PI 1A1, PI 3-1, and PI 5-1 alter the capacity of σ1 to form oligomers, we assessed the mobility of the σ1 protein of each of these strains (in addition to PI 2A1 as a control) in SDS-polyacrylamide gels after incubating virions in sample buffers in which the pH was varied from 6.0 to 10.5 (Fig. 2). In these experiments, the mobilities of σ1 proteins of T3D and PI 2A1 were identical. High-molecular-weight protein bands (molecular weight, ~200,000) consistent with oligomers of σ1 could be detected when virions of these viruses were electrophoresed after incubation in sample buffers of pH ≥7.0. In contrast, σ1 protein of PI 1A1 migrated as a low-molecular-weight species (molecular weight, ~49,000) consistent with monomers of σ1 after incubation in sample buffers at all pHs tested, including pH 10.5. Stability of σ1 oligomers of L/C, PI 3-1, and PI 5-1 was intermediate to that of σ1 oligomers of T3D and PI 1A1. High-molecular-weight σ1 bands were detected after incubation in sample buffers of pH ≥8.0; low-molecular-weight bands were detected after incubation in sample buffers of pH ≤7.0. Therefore, mutations in PI virus σ1 protein result in either of two classes of alterations in stability of σ1 oligomers: (i) unstable σ1 oligomers at pH 6.0 to 10.5 (PI 1A1) or (ii) stable σ1 oligomers at pH ≥8.0 and unstable σ1 oligomers at pH ≤7.0 (L/C, PI 3-1, and PI 5-1). The σ1 proteins of T2J and the PI viruses with S1 genes derived from T2J, L/T2 + C, and L/T2 + T3 did not migrate as oligomers under these conditions (data not shown).

In addition to varying sample buffer pH, stability of σ1 oligomers in SDS-polyacrylamide gels is influenced by the concentration of divalent cations in the sample buffer (31). Low divalent cation concentrations favor migration of σ1 as an oligomer, whereas high concentrations favor migration of σ1 as a monomer. To determine whether mutations in PI virus σ1 protein affect stability of σ1 oligomers in conditions of varying divalent cation concentrations, wt and PI virus virions were incubated in sample buffer under conditions favoring stability of σ1 oligomers (pH 10.5) with the $CaCl_2$ concentration adjusted to either 0 or 15 mM (Fig. 3). T3D, L/C, and PI 2A1 σ1 proteins migrated as 200,000-molecular-weight bands after incubation in sample buffer containing either 0 or 15 mM $CaCl_2$. PI 1A1 σ1 migrated as a 49,000-molecular-weight band after incubation in the presence or absence of $CaCl_2$. PI 3-1 and PI

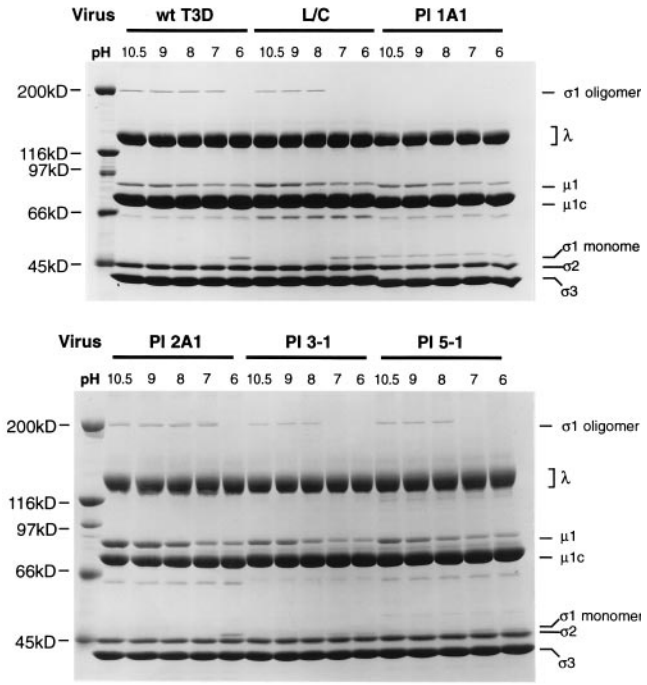

FIG. 2. Effect of pH on migration of σ1 protein in SDS-polyacrylamide gels. Purified virions of T3D and PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 were disrupted in sample buffer containing 62.5 mM Tris (pH 6 to 10.5), 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol. Virus samples were incubated at 65°C for 5 min prior to electrophoresis in a 5 to 15% gradient polyacrylamide gel. Molecular weight standards and viral proteins are labeled. Oligomer and monomer bands of σ1 are indicated.

5-1 σ1 proteins migrated as 200,000-molecular-weight bands after incubation in sample buffer lacking $CaCl_2$ and as 49,000-molecular-weight bands after incubation in sample buffer containing 15 mM $CaCl_2$. Therefore, with the exception of PI virus L/C, mutations in the tail domain of PI virus σ1 protein decrease the stability of σ1 oligomers after incubation of virions in sample buffer containing $CaCl_2$. These results are in agreement with our studies of the effect of pH on oligomer stability and suggest that there are two classes of alterations in stability of σ1 oligomers: (i) unstable oligomers in the absence of $CaCl_2$ (PI 1A1) and (ii) stable oligomers in the absence of $CaCl_2$ and unstable oligomers in the presence of 15 mM $CaCl_2$ (PI 3-1 and PI 5-1).

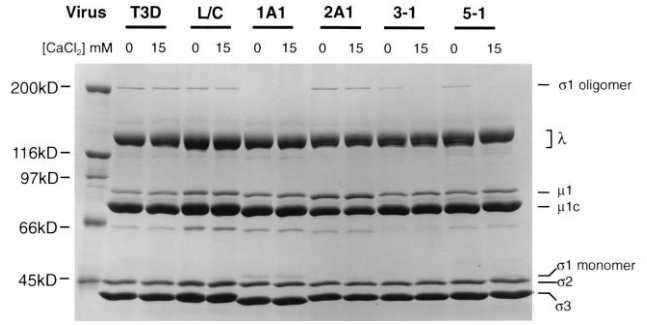

FIG. 3. Effect of addition of 15 mM $CaCl_2$ to the sample buffer on migration of σ1 protein in SDS-polyacrylamide gels. Purified virions of T3D and PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 were disrupted in sample buffer containing 62.5 mM Tris (pH 10.5), 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, and either 0 or 15 mM $CaCl_2$. Virus samples were incubated at 65°C for 5 min prior to electrophoresis in a 5 to 15% gradient polyacrylamide gel. Molecular weight standards and viral proteins are labeled. Oligomer and monomer bands of σ1 are indicated.

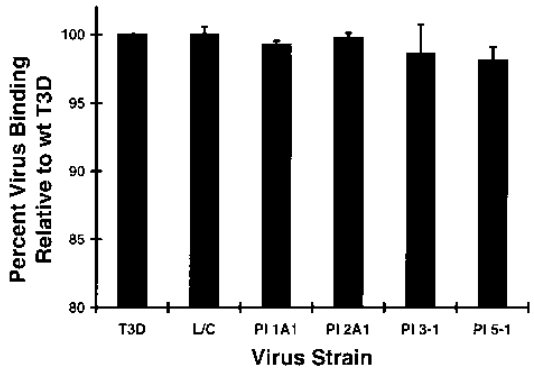

FIG. 4. Binding of wt and PI viruses to L cells. Virions of wt strain T3D and PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 were adsorbed to L cells at 4°C for 1 h and then incubated with rabbit antireovirus antiserum, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Virus binding was quantitated by fluorescence-activated cytometric analysis. Experiments were performed in triplicate, and the results are presented as the mean percent virus binding relative to the binding of T3D. Error bars indicate standard deviations of the means.

The capacity of wt and PI viruses to attach to cells. Amino acid sequences important for attachment of type 3 reovirus to L cells are contained in the σ1 head (20, 49, 56), while sequences important for HA are contained in the σ1 tail (15, 36). To determine whether mutations in PI virus σ1 protein affect the binding of PI viruses to L cells, we used fluorescence-activated cytometric analysis to assess the binding of wt virus T3D and PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 to L-cell receptors (Fig. 4). No differences were detected in the attachment of wt and PI viruses to L cells, suggesting that mutations affecting stability of σ1 oligomers do not alter PI virus binding to L-cell receptors.

To assess whether mutations in PI virus σ1 protein influence the capacity of the PI virus strains to produce HA, a property dependent on σ1 binding to sialic acid (5, 40, 41), we determined HA titers of wt T3D and PI viruses L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 in assays using bovine erythrocytes (Table 3). With the exception of PI 2A1, HA titers of the PI viruses tested varied from 4- to 16-fold less than that of T3D. The HA titer of PI 2A1, which does not contain a mutation in the deduced amino acid sequence of its σ1 protein (Table 2), is identical to that of T3D. Therefore, while mutations in PI virus σ1 protein do not affect PI virus binding to L cells, they do affect the capacity of these viruses to bind sialic acid-containing receptors on erythrocytes.

TABLE 3. Capacity of wt and PI viruses to produce HA

Virus strain	HA titer[a]
T3D	1,024
L/C	256
PI 1A1	64
PI 2A1	1,024
PI 3-1	64
PI 5-1	256

[a] Number of HA units per 2×10^{11} viral particles. One HA unit equals the number of particles sufficient to produce HA. The results are presented as the mean HA titers for three independent experiments.

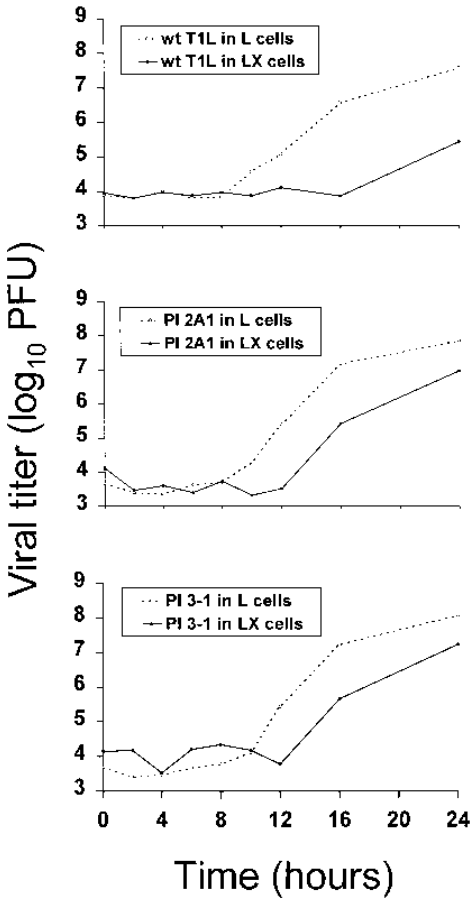

FIG. 5. Growth of reovirus strains T1L, PI 2A1, and PI 3-1 in wt and cured cells. Monolayer cultures of wt L cells and cured LX cells (5×10^5 cells) were infected with viral strains at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium was added, and the cells were incubated at 37°C for the times shown. Cell lysates were titrated on L-cell monolayers by plaque assay. Experiments were performed in duplicate, and the results are presented as the mean viral yields.

Growth of PI 2A1 in cured cells is determined by the viral S4 gene. The absence of mutations in the deduced amino acid sequence of σ1 protein of PI 2A1 was unexpected and suggested that genes other than S1 might confer the capacity of some PI viruses to grow better than wt virus in cells cured of persistent infection. To identify viral genes associated with growth of PI 2A1 in cured cells, we isolated reassortant viruses from mixed infections of wt strain T1L and PI 2A1. As shown in Fig. 5, PI 2A1 produces approximately 100-fold-greater yields of viral progeny than T1L in cured LX cells after a 24-h period of viral growth. T1L × PI 2A1 reassortant viruses were used to infect L cells and LX cells, and viral yields were determined by plaque assay after 24 h (Table 4). Yields in LX cells were divided by those in L cells (LX/L ratio) to normalize the growth of each PI virus in LX cells. Reassortant viruses containing an S4 gene derived from PI 2A1 had LX/L ratios of >0.1, whereas those with an S4 gene from T1L had LX/L ratios of <0.1. No other reovirus genes were associated with LX/L ratios of >0.1, which suggests that mutations in the S4 gene determine the capacity of PI 2A1 to grow better than wt in cured cells. Therefore, these findings indicate that in some cases, mutations in the S4 gene are associated with efficiency of PI virus growth in cured cells.

Growth of PI 3-1 in cured cells is determined by the viral S1 gene. To determine whether the S1 gene segregates with growth in cured cells of an additional PI virus that contains an S1 gene mutation, we isolated reassortant viruses from mixed infections of T1L and PI 3-1. Like PI 2A1, PI 3-1 produces approximately 100-fold-greater yields than T1L in cured LX cells after 24 h of viral growth (Fig. 5). L cells and LX cells were infected with T1L × PI 3-1 reassortant viruses, and viral yields were determined by plaque assay after a 24-h period of viral growth (Table 5). Reassortant viruses containing an S1 gene derived from PI 3-1 had LX/L ratios of >0.09, whereas those with an S1 gene from T1L had LX/L ratios of <0.09. No other reovirus genes were associated with LX/L ratios of >0.09, which suggests that mutations in the S1 gene determine the capacity of PI 3-1 to grow better than wt in cured cells. Thus, depending on the PI virus, mutations selected during persistent reovirus infection in either the S1 or S4 genes can be associated with the capacity of PI viruses to grow better than wt in mutant cells cured of persistent infection.

DISCUSSION

S1 gene mutations are selected during persistent reovirus infections of L cells. During maintenance of persistent reovirus infections of L cells, viral mutations that promote the capacity of PI viruses to grow better than wt viruses in cells cured of persistent infection are selected (1, 17). In previous work, the S1 gene was shown to be a determinant of the capacity of a prototype PI virus, L/C, to grow in cured cells (27). We now show that the S1 gene segregates with growth in cured cells of an additional PI virus, PI 3-1. To identify which of the S1 gene products is associated with growth in cured cells and to elucidate mechanisms by which mutations in S1 promote maintenance of persistent reovirus infection, we determined the S1 gene nucleotide sequences of seven independent PI viruses. Few mutations were observed in the S1 genes of the PI viruses examined despite the fact these viruses were isolated after a minimum of 89 days in culture. S1 gene mutations in PI viruses L/C, L/T2 + C, L/T2 + T3, PI 1A1, PI 3-1, and PI 5-1 resulted in changes in the deduced amino acid sequence of σ1 protein, and S1 mutations in L/C, PI 1A1, PI 2A1, PI 3-1, and PI 5-1 resulted in changes in the deduced amino acid sequence of σ1s. Our findings that growth in cured cells of PI 2A1 × T1L reassortants segregates with the S4 gene and that S1 gene mutations in L/T2 + C and L/T2 + T3 do not result in mutations in σ1s strongly suggest that mutations in σ1, and not σ1s, are important for growth of PI viruses in cured cells. This hypothesis is also consistent with previous studies which suggest that steps in viral entry, which occur prior to synthesis of σ1s, are targets for viral and cellular mutations selected during persistent reovirus infection (17, 55).

Entry of reovirus into cells requires proteolytic processing of viral outer-capsid proteins, leading to generation of ISVPs (12, 46, 48). During virion-to-ISVP processing, the σ3 protein is degraded, viral attachment protein σ1 undergoes a conformational change, and the μ1/μ1C proteins are cleaved generating particle-associated fragments μ1δ/δ and φ (39). Cellular mutants selected during persistent infection do not support one or more steps in viral entry leading to formation of ISVPs (17, 55). Since PI viruses have evolved the capacity to infect these mutant cells, these viruses likely manifest alterations in viral outer-capsid proteins involved in viral disassembly. In concordance with this prediction, relevant mutations in PI viruses were found to map to the σ1-encoding S1 gene and the σ3-encoding S4 gene. Results from the present study provide insight into mechanisms by which mutations in σ1 protein

TABLE 4. Growth of T1L × PI 2A1 reassortant viruses in cured LX cells and wt L cells

Virus strain	Origin of gene segment[a]										Yield in[b]:		LX/L ratio[c]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	LX cells	L cells	
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	1.9×10^6	2.2×10^8	0.009
PI 2A1	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	7.6×10^7	3.8×10^8	0.20
JW35	PI	PI	PI	wt	wt	wt	PI	wt	wt	PI	8.7×10^7	1.1×10^8	0.79
JW24	PI	PI	PI	PI	PI	wt	PI	PI	wt	PI	3.0×10^6	5.9×10^6	0.51
JW34	wt	wt	wt	wt	wt	PI	wt	wt	PI	PI	1.6×10^7	5.2×10^7	0.31
JW36	PI	PI	PI	PI	PI	PI	PI	PI	wt	PI	3.6×10^6	1.3×10^7	0.28
JW56	PI	PI	PI	wt	wt	PI	wt	PI	wt	PI	8.6×10^6	4.4×10^7	0.20
JW33	PI	PI	PI	wt	PI	wt	wt	PI	PI	PI	1.2×10^6	6.4×10^6	0.19
JW222	PI	PI	wt	PI	PI	PI	wt	PI	PI	PI	2.0×10^7	1.3×10^8	0.15
JW22	PI	PI	PI	PI	wt	PI	wt	wt	PI	PI	8.2×10^6	6.8×10^7	0.12
JW1	PI	PI	PI	wt	wt	PI	wt	PI	wt	PI	7.6×10^6	6.8×10^7	0.11
JW101	wt	PI	wt	wt	wt	wt	wt	PI	PI	wt	8.4×10^5	1.7×10^7	0.049
JW12	PI	PI	PI	wt	wt	wt	wt	wt	wt	wt	2.3×10^6	4.9×10^7	0.047
JW23	wt	PI	wt	wt	wt	wt	wt	wt	wt	wt	2.8×10^6	6.2×10^7	0.045
JW260	PI	PI	wt	PI	PI	PI	wt	PI	PI	wt	3.7×10^5	1.3×10^7	0.028
JW52	wt	wt	wt	wt	wt	wt	wt	PI	PI	wt	2.4×10^6	1.3×10^8	0.018

[a] Parental origin of each gene segment in the reassortant strains. wt, gene segment derived from T1L; PI, gene segment derived from PI 2A1.
[b] Cured LX cells or wt L cells (5×10^5 cells) were infected with virus strains at an MOI of 2 PFU per cell and incubated at 37°C for 24 h. Cells were frozen and thawed twice and titrated on L-cell monolayers by plaque assay. Experiments were performed in duplicate, and the results are presented as mean viral yields in each cell type.
[c] The mean viral yield in LX cells was divided by that in L cells to calculate an LX/L ratio for each virus strain.

facilitate PI virus growth in mutant cells. Mechanisms by which mutations in σ3 protein promote viral growth in mutant cells are the subject of current work in our laboratory.

Mutations in PI virus σ1 proteins affect stability of σ1 oligomers. Amino acid sequences in the σ1 tail are predicted to form a tandem arrangement of α-helix and β-sheet structural motifs, whereas sequences in the σ1 head are predicted to assume a more complex globular structure (16, 37). Mutations in σ1 proteins of PI viruses L/C, PI 1A1, PI 3-1, and PI 5-1 are contained in a long region of sequence (~150 residues) in the σ1 tail conforming to a heptad repeat pattern of apolar residues (7, 16, 19, 22, 37) (Fig. 1). Amino acid sequences in such heptad repeat patterns are predicted to form α-helical coiled coils (35), and the long heptad repeat region in σ1 is important for stability of σ1 oligomers (30). Current data suggest that the oligomeric species of σ1 is either a trimer (30, 33, 47) or a tetramer (8, 22). In the former model, σ1 oligomers are proposed to be formed from a three-stranded homotrimer (47); in the latter, oligomers of σ1 are suggested to be formed from a parallel pair of σ1 dimers (22). In either model, sequences conforming to a heptad-repeat pattern of apolar residues are likely to provide stability to the higher-order structure through hydrophobic interactions over an extended region of sequence (Fig. 6).

We assessed the stability of oligomers of PI virus σ1 proteins in SDS-polyacrylamide gels, using conditions in which pH and divalent cation concentrations were varied in the sample buffer prior to electrophoresis. Stability of oligomers of PI 2A1 σ1, which does not contain a mutation, was identical to stability of oligomers of wt T3D. However, mutations in σ1 proteins of L/C, PI 1A1, PI 3-1, and PI 5-1 were found to decrease the stability of σ1 oligomers. The deduced amino acid sequence of PI 1A1 σ1 contains a single substitution of an arginine for a leucine at amino acid 91. Leucine 91 occupies a position in the apolar repeat pattern corresponding to a d position in the helical wheel projection shown in Fig. 6. The substitution of a

TABLE 5. Growth of T1L × PI 3-1 reassortant viruses in cured LX cells and wt L cells

Virus strain	Origin of gene segment[a]										Yield in[b]:		LX/L ratio[c]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	LX cells	L cells	
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	1.0×10^5	1.4×10^7	0.007
PI 3-1	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	1.5×10^7	1.4×10^8	0.11
LD10	PI	PI	wt	wt	wt	PI	PI	PI	wt	PI	1.4×10^7	3.4×10^7	0.42
LD134	wt	wt	wt	wt	wt	PI	PI	wt	wt	PI	7.0×10^6	3.4×10^7	0.21
LD145	wt	wt	wt	wt	wt	wt	PI	wt	wt	wt	8.0×10^6	5.0×10^7	0.16
LD110	PI	PI	wt	PI	PI	PI	PI	PI	wt	wt	1.5×10^6	1.4×10^7	0.11
LD131	wt	wt	wt	wt	wt	wt	PI	PI	wt	wt	3.2×10^6	3.0×10^7	0.11
LD123	wt	PI	wt	wt	PI	PI	PI	PI	wt	wt	2.7×10^6	2.6×10^7	0.10
LD138	wt	wt	wt	PI	PI	wt	PI	PI	wt	PI	5.8×10^6	6.3×10^7	0.093
LD4	wt	PI	wt	wt	wt	PI	wt	wt	wt	wt	6.6×10^5	9.5×10^6	0.070
LD144	wt	wt	wt	PI	PI	wt	wt	PI	PI	PI	2.1×10^6	4.7×10^7	0.045
LD127	wt	wt	wt	wt	wt	wt	wt	wt	PI	wt	3.1×10^5	2.6×10^7	0.012

[a] Parental origin of each gene segment in the reassortant strains. wt, gene segment derived from T1L; PI, gene segment derived from PI 3-1.
[b] Cured LX cells or wt L cells (5×10^5 cells) were infected with virus strains at an MOI of 2 PFU per cell and incubated at 37°C for 24 h. Cells were frozen and thawed twice and titrated on L-cell monolayers by plaque assay. Experiments were performed in duplicate, and the results are presented as mean viral yields in each cell type.
[c] The mean viral yield in LX cells was divided by that in L cells to calculate an LX/L ratio for each virus strain.

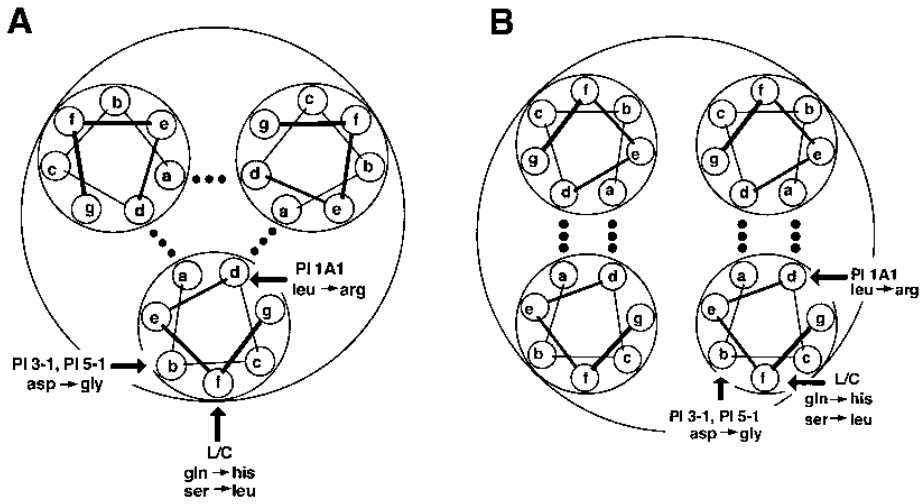

FIG. 6. Mutations in PI virus σ1 protein are within a region of sequence predicted to form α-helical coiled coil. Shown are schematic diagrams of a σ1 homotrimer as proposed by Strong et al. (47) (A) and a σ1 tetramer formed from a dimer of α-helical coiled-coil dimers as proposed by Fraser et al. (22) (B). The dimensions of an ideal α-helical coiled-coil structure (~3.5 residues per turn) dictate that apolar residues in positions a and d of a heptad forming an α-helix occur on the same side of a cylinder defined by the helix. Interactions between apolar residues of adjacent α-helices in a coiled coil, indicated by dashed lines, provides the major force stabilizing dimers or trimers of α-helices. The residues at other heptad positions (b, c, e, f, and g) project into the surrounding solution and are usually polar. Interactions between polar residues of adjacent coiled-coil dimers are suggested to stabilize tetramers (22).

large basic residue for an apolar residue could disrupt hydrophobic interactions between adjacent α-helices, leading to instability of σ1 oligomers, regardless of subunit stoichiometry. Consistent with this prediction, we did not detect oligomers of PI 1A1 σ1 protein under any of the electrophoresis conditions in this study. The deduced σ1 amino acid sequences of PI 3-1 and PI 5-1 contain an aspartate-to-glycine change at amino acid 124, which corresponds to a b position in the apolar repeat motif (Fig. 6). Glycine residues destabilize α-helices (13) and might be expected to affect stability of σ1 oligomers on that basis. Alternatively, substitution of glycine for aspartate at amino acid 124 might affect intermolecular hydrophilic interactions, resulting in instability of σ1 oligomers formed from dimers of σ1 dimers.

The stability of oligomers of L/C virus σ1 protein was found to be intermediate to that of oligomers of the other PI viruses studied. Oligomers of L/C σ1 were less stable than oligomers of wt T3D at high pH but as stable as oligomers of T3D at dilute concentrations of $CaCl_2$. The deduced amino acid sequence of L/C σ1 contains two mutations, glutamine to histidine at amino acid 51 and serine to leucine at amino acid 244. The amino terminus of these mutations occurs within the long heptad repeat region at an f position in the apolar repeat pattern (Fig. 6), and this mutation might lead to instability of σ1 oligomers by mechanisms similar to those postulated to decrease stability of oligomers of PI 3-1 and PI 5-1 σ1 proteins.

We hypothesize that changes in the stability of σ1 oligomers might alter a conformational change in σ1 that occurs during entry of reovirus into cells. Such a conformational change might be required to target the virus-receptor complex to an endocytic compartment where proteolytic processing of the outer capsid occurs or facilitate proteolysis of outer-capsid proteins by endocytic proteases. Alternatively, mutations affecting the stability of σ1 oligomers might affect later steps in viral entry, such as interaction of processed ISVPs with vacuolar membranes or activation of the viral transcriptase. Enhancement of any of these entry steps would be anticipated to augment viral growth in cells manifesting blocks to viral disassembly, like those selected during persistent reovirus infection. Of note, the carboxy-terminal mutation in L/C σ1 is contained in a region of the protein predicted to form α-helix, which separates two larger regions of predicted β-sheet (16, 37) (Fig. 1). This region of sequence has been identified as a site of flexibility within σ1 (22), and it is possible that the mutation at amino acid 244 in L/C σ1 protein affects conformational changes in σ1 that occur during virion-to-ISVP processing independent of effects on oligomer stability.

Our observation that oligomers of PI virus σ1 protein are less stable than oligomers of wt σ1 at increased pH suggests that the conformational change in σ1 during viral disassembly (18, 23, 36) is pH dependent. Acid-dependent conformational changes in viral attachment proteins during viral entry have been reported for several viruses, including influenza virus (11), Semliki Forest virus (28, 51), and tick-borne encephalitis virus (4). In addition, pH-sensitive events involving viral attachment proteins have been shown to be altered in some types of persistent infections. Mutations in the spike glycoprotein of mouse hepatitis virus type 4 selected during persistent infection result in alterations in the pH optimum of membrane fusion (24). Sequence analysis of the mutant spike glycoprotein revealed three point mutations in a heptad repeat region of the transmembrane cleavage fragment of the protein, including a leucine-to-arginine change at a d position in the heptad repeat pattern.

The single point mutation in the σ1 protein of L/T2 + C and the amino terminus of two mutations in the σ1 protein of L/T2 + T3 were contained in a region of the protein important for anchoring σ1 to the virion (32, 34). These mutations might promote growth in cured cells by altering interactions with core-spike protein λ2 during viral disassembly. These mutations also might affect the stability of σ1 oligomers; however, this could not be assessed. The S1 genes of L/T2 + C and L/T2 + T3 were derived from T2J, and oligomers of T2J σ1 were not detected under the electrophoresis conditions used in these experiments. In addition to point mutations, the deduced amino acid sequences of L/T2 + C and L/T2 + T3 σ1 proteins contained identical eight-residue deletions in the σ1 tail. The deleted sequences correspond to a single β-strand in a region

of σ1 sequence predicted to form an eight-stranded β-sheet (37). The importance of this region is not apparent from current knowledge of the structure and function of the T2J σ1 protein; however, it is possible that sequences in this region contribute to oligomer stability or form a domain that interacts with other components of the viral outer capsid during virion-to-ISVP processing.

L-cell binding by PI viruses derived from T3D was equivalent to that of T3D; however, the capacity of PI viruses with less stable σ1 oligomers to produce HA was decreased. Altered stability of σ1 oligomers might decrease the affinity of σ1 for sialic acid, the carbohydrate bound by type 3 reovirus on erythrocytes (5, 40, 41), or decrease the capacity of σ1 to cross-link sialylated receptors required for the agglutination of erythrocytes.

PI viruses are disassembly mutants of reovirus. Our results suggest that PI reoviruses are disassembly mutants that contain mutations in either the σ1 or σ3 proteins. We postulate that these mutations enhance the processing of PI virus outer-capsid proteins and thereby facilitate PI virus growth in mutant cells that do not efficiently support viral disassembly. Our studies of PI virus σ1 proteins suggest that mutations in the σ1 tail lead to alterations in virion-to-ISVP processing and enhance the capacity of these viruses to adapt to growth in carrier cultures. These results suggest that viruses selected during persistent reovirus infection will be particularly useful for defining interactions between viral outer-capsid proteins during reovirus disassembly.

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