Molecular Mechanisms of Persistent Infection by Reovirus

T.S. DERMODY

1 Introduction

Many cytolytic animal viruses are capable of establishing persistent infections of cultured cells. For such persistent infections to be maintained interactions between virus and cell must be modulated such that a less cytopathic virus-host relationship is established (reviewed in AHMED et al. 1996). Variant viruses may be selected that are attenuated in cytolytic potential, or variant cells may be selected that are less permissive for viral replication. In some cases, however, viruses and cells coevolve during persistent infection, such that selection of virus-resistant cells leads to counterselection of highly infective mutant viruses that can grow in resistant cells.

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 in persistent infections caused by several viruses, including coronavirus (GALLAGHER et al. 1991;

Departments of Pediatrics, and Microbiology and Immunology, and Elizabeth B. Lamb Center for Pediatric Research, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
(E-mail: terry.dermody@mcmail.vanderbilt.edu)

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CHEN and BARIC 1996), poliovirus (KAPLAN et al. 1989; BORZAKIAN et al. 1992), reovirus (DERMODY et al. 1993; WETZEL et al. 1997a), and rotavirus (MRUKOWICZ et al., 1998). Identification of the specific strategies used by viruses to overcome cellular blocks to viral entry has provided new insights into mechanisms by which viruses enter cells.

2 Persistent Reovirus Infections are Carrier Cultures That Require Horizontal Cell-to-Cell Transmission for Their Propagation

Although usually cytolytic in cell culture, mammalian reoviruses can establish persistent infections of many types of cells, including human embryonic fibroblast cells (BELL et al. 1966), Burkitt's lymphoma cells (LEVY et al. 1968), Chinese hamster ovary (CHO) cells (TABER et al. 1976), murine L929 (L) cells (AHMED and GRAHAM 1977; AHMED and FIELDS 1982; BROWN et al. 1983; DERMODY et al. 1993), murine B- and T-cell hybridomas (MATSUZAKI et al. 1986; DERMODY et al. 1995), murine 3T3 cells (VERDIN et al. 1986), Madin-Darby canine kidney (MDCK) cells (MONTGOMERY et al. 1991), murine SC1 cells (DANIS et al. 1993), and murine erythroleukemia (MEL) cells (WETZEL et al. 1997a). Cell cultures persistently infected with reovirus produce high titers of virus for long periods of time, and a majority of cells in the cultures shows evidence of viral infection (TABER et al. 1976; AHMED and GRAHAM 1977; VERDIN et al. 1986; DERMODY et al. 1993; WETZEL et al. 1997a). Anti-reovirus antibody treatment of persistent infections of CHO cells (TABER et al. 1976), L cells (AHMED et al. 1981; DERMODY et al. 1993), B-cell hybridomas (DERMODY et al. 1995), and MEL cells (WETZEL et al. 1997a) results in cure of persistent infection.

The observation that antibody treatment can cure cell cultures persistently infected with reovirus suggests that persistent reovirus infections are maintained by horizontal transmission of virus between cells (MAHY 1985). Antibodies are believed to neutralize viral infectivity by blocking early steps in viral replication, such as attachment, penetration, and disassembly (reviewed in WHITTON and OLDSTONE 1996). It is likely that neutralizing anti-reovirus antibodies block one or more of these early steps, which would interrupt horizontal viral transmission and result in cure of persistent infection. It is possible that some component of vertical viral transmission occurs during persistent reovirus infection of cultured cells. However, the rapid decrease in viral titer during antibody treatment (DERMODY et al. 1993, 1995; WETZEL et al. 1997a) suggests that horizontal transmission is the primary mechanism of viral spread in these cultures.

3 Distinct Phases of Persistent Reovirus Infection Have Been Identified

Persistent reovirus infections of L cells have been used as a useful model system to define mechanisms that foster long-term propagation of persistent viral infections.

From studies of persistently infected L-cell cultures, a general paradigm has emerged which holds that there are two distinct phases of persistent infection: establishment and maintenance. Establishment of persistent infection is characterized by intense cell crises in which only a few colonies of cells survive. Maintenance is characterized by stable cell growth and continuous production of substantial titers of infectious virus (ca. 1×10^6–1×10^8 plaque-forming units per milliliter of culture supernatant) for prolonged periods of passage, in some cases in excess of 3 years (T.S. DERMODY, unpublished observations).

Establishment of persistent reovirus infections of L cells occurs when infection is initiated with virus stocks passaged serially at high multiplicity of infection (AHMED and GRAHAM 1977; AHMED and FIELDS 1982; BROWN et al. 1983; DERMODY et al. 1993). Such stocks contain a variety of viral mutants (AHMED et al. 1980, 1983), and some of these mutants may facilitate establishment of persistent infection. In an early study of persistent reovirus infection, mutations in the viral S4 gene segment, which encodes outer-capsid protein σ3, were suggested to be important for establishment of persistent infection (AHMED and FIELDS 1982). In this study L cells were coinfected with a low-passage stock of reovirus strain type 2 Jones (T2J), which causes lytic infections, and a high-passage stock of strain type 3 Dearing (T3D), which causes persistent infections, under conditions to promote persistent infection. It was reasoned that gene segments selected from the T3D high-passage stock during the coinfection would identify viral mutations required for establishment of persistent infection. In three independent coinfections, the S4 gene segment was the only gene found to be consistently selected from the T3D high-passage stock (AHMED and FIELDS 1982), arguing that mutations in S4 are required for establishment of persistent infection. However, it is also possible that the T3D S4 gene confers a selective advantage over the T2J S4 gene in mixed infections of L cells, rather than mediating establishment of persistent infection.

Not all reovirus strains generate mutations capable of establishing persistent infection during high passage. Stocks of T3D, but not strain type 1 Lang (T1L), passaged at high multiplicity of infection contain deletions and other mutations and readily establish persistent infections (BROWN et al. 1983). Reassortant viruses containing a T3D L2 gene segment mediate these properties (BROWN et al. 1983), suggesting that L2 gene product λ2 is important for the generation of mutations that allow persistent infection to be established. The λ2 protein is a major component of the reovirus core (RALPH et al. 1980; DRYDEN et al. 1993), and λ2 serves as the viral guanylyltransferase (CLEVELAND et al. 1986). Therefore it is possible that λ2-mediated functions in viral RNA synthesis are the basis for differences in the mutation frequency exhibited by T1L and T3D (BROWN et al. 1983).

Other data indicate that the type of host cell determines whether persistent infection is established. Persistent infections of 3T3 cells (VERDIN et al. 1986), CHO cells (TABER et al. 1976), MDCK cells (MONTGOMERY et al. 1991), SC1 cells (DANIS et al. 1993), and MEL cells (WETZEL et al. 1997a) can be established using low-passage reovirus stocks, which lack the capacity to establish persistent infections of L cells (AHMED and FIELDS 1982; WETZEL et al. 1997a). In some cases the capacity of cells to support establishment of persistent infection is linked to resistance to

reovirus-induced inhibition of cellular protein synthesis (DUNCAN et al. 1978; DANIS et al. 1993). In other cases establishment of persistent infection is favored by the inability of host cells to efficiently support acid-dependent proteolytic disassembly of reovirus virions during viral entry. Treatment of L cells with the weak base ammonium chloride, which blocks acid-dependent proteolysis of reovirus virions (STURZENBECKER et al. 1987), leads to establishment of persistent infections using low-passage reovirus stocks (CANNING and FIELDS 1983). The capacity of MEL cells to promote establishment of persistent infection is also linked to an inability to efficiently support viral disassembly (WETZEL et al. 1997a). These observations suggest that blocks to reovirus entry, either pharmacological or endogenous, favor persistent over lytic infections of cultured cells.

4 Mutations in Cells and Viruses Affecting Viral Entry Coevolve During the Maintenance Phase of Persistent Reovirus Infection

Coevolution of viruses and cells during persistent reovirus infection has been documented in studies of persistent infections of L cells (AHMED et al. 1981; DERMODY et al. 1993) and MEL cells (WETZEL et al. 1997a). Viruses selected during maintenance of these persistent infections (termed PI viruses) grow better than wild-type (wt) viruses in cells cured of persistent infection (Fig. 1), indicating that mutant viruses are selected during persistent infection. Similarly, wt viruses grow better in parental cells than in cured cells, indicating that mutant cells are also selected in these cultures (Fig. 1). Insight into the nature of mutations selected in cells and viruses during persistent reovirus infection was first suggested by electron micrographic analysis of persistently infected and cured L cells (AHMED et al. 1981; SHARPE and FIELDS 1983). Both types of cells accumulate large numbers of vacuoles that resemble lysosomes (Fig. 2). These findings led to the hypothesis that mutations in cells affect steps in reovirus entry dependent on proteolysis of the viral outer capsid in vacuoles of the endocytic compartment.

Reovirus entry is initiated by stable attachment of the virus to the surface of the host cell. The σ1 protein, which is encoded by the S1 gene segment, serves as the reovirus attachment protein (WEINER et al. 1980; LEE et al. 1981). The σ1 protein is a fibrous protein (FURLONG et al. 1988; FRASER et al. 1990) located at the 12 vertices of the virion icosahedron (FURLONG et al. 1988; DRYDEN et al. 1993). Native σ1 protein forms an oligomer, and current data suggest that the oligomeric species of σ1 is either a trimer (LEONE et al. 1991a, 1992; STRONG et al. 1991) or a tetramer (BASSEL-DUBY et al. 1987; FRASER et al. 1990). Following viral attachment, virions are observed by electron microscopy in clathrin-coated pits, which suggests that virion uptake occurs by receptor-mediated endocytosis (BORSA et al. 1979, 1981; STURZENBECKER et al. 1987; RUBIN et al. 1992). Within late endosomes or lysosomes, viral outer-capsid proteins σ3 and μ1/μ1C are subject to proteolysis by cellular proteases, resulting in generation of infectious subvirion particles

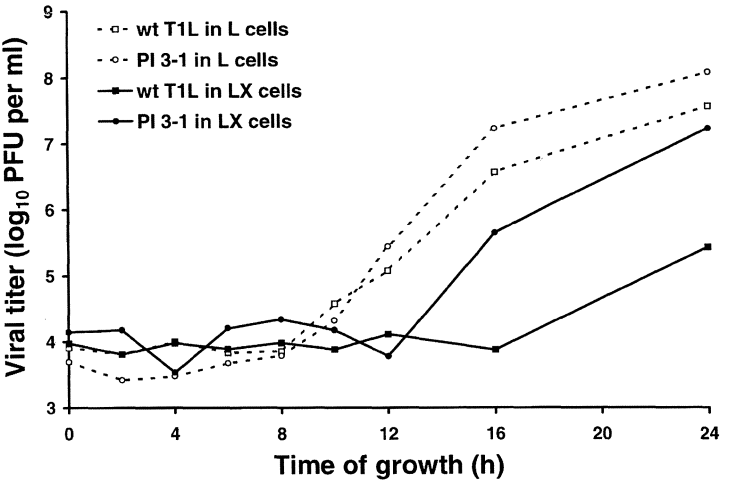

Fig. 1. Growth of wt virus T1L and PI virus PI 3-1 in parental L cells and cured LX cells. Monolayers of cells (5×10^5 cells) were infected with either T1L or PI 3-1 at an MOI of 2 PFU per cell. After a 1-h adsorption period the inoculum was removed, fresh medium was added, and the cells were incubated at 37°C for the time, shown. 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 titers for two independent experiments. (Adapted from WILSON et al. 1996)

(ISVPs) (CHANG and ZWEERINK 1971; SILVERSTEIN et al. 1972; BORSA et al. 1981; STURZENBECKER et al. 1987). During this process σ3 is degraded and lost from virions, viral attachment protein σ1 undergoes a conformational change, and μ1/μ1C is cleaved to form particle-associated fragments μ1δ/δ and φ (reviewed in NIBERT et al. 1996). ISVPs generated in the endocytic compartment are probably identical to those generated either in the intestinal lumen of perorally infected mice (BODKIN et al. 1989; BASS et al. 1990) or in vitro by treatment of virions with chymotrypsin or trypsin (CHANG and ZWEERINK 1971; SILVERSTEIN et al. 1972; BORSA et al. 1981; STURZENBECKER et al. 1987; NIBERT et al. 1995). Intracellular proteolysis of σ3 and μ1 is an acid-dependent process as treatment of cells with the weak base ammonium chloride (STURZENBECKER et al. 1987; DERMODY et al. 1993) or inhibitors of the vacuolar proton ATPase, such as bafilomycin or concanamycin A (MARTINEZ et al. 1996), blocks infection by virions but not by ISVPs.

The availability of in vitro generated reovirus disassembly intermediates has facilitated conclusive demonstration that mutant cells are altered in their capacity to support viral entry. Cured cells do not support efficient growth of wt virus when infection is initiated with virions, but do so when infection is initiated with in vitro generated ISVPs (DERMODY et al. 1993) (Fig. 3). These findings indicate that mutant cells selected during persistent reovirus infection do not support steps in viral entry leading to generation of ISVPs. The mechanisms by which mutant cells block steps in reovirus entry are not known; however, it is possible that mutant cells are altered in their capacity to bind virions, internalize virions by receptor-mediated endocytosis, mediate acid-dependent disassembly of the viral outer capsid, or

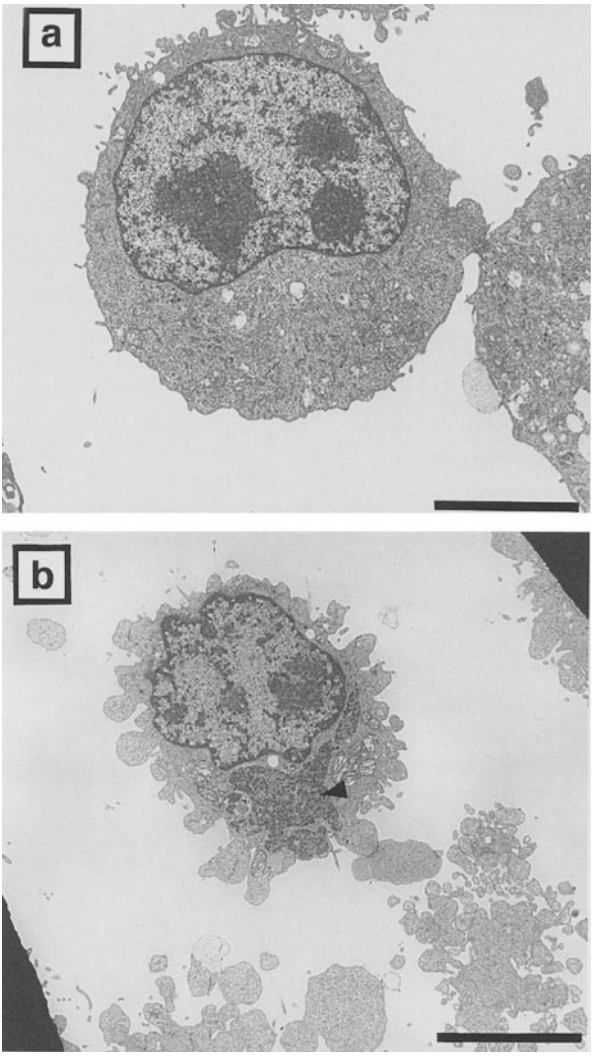

Fig. 2a–d. Morphology of uninfected L cells, persistently infected L cells, and cured LX cells. **a** Uninfected L cells. **b** Persistently infected L cells showing large inclusion of progeny virions (*arrowhead*). **c** Persistently infected L cells showing membrane-bound organelles (*arrows*). **d** Cured LX cells showing membrane-bound organelles (*arrows*). *Bars*, 5μm

facilitate penetration of the viral core into the cytoplasm. Cells manifesting alterations in endocytic function analogous to those selected during persistent reovirus infection are also observed after selection for resistance to diphtheria toxin. Organelles in the central vacuolar system of diphtheria-toxin-resistant CHO cells are altered in their acidification capacity (MERION et al. 1983). Therefore changes in endocytic uptake and proteolytic processing might be common mechanisms for

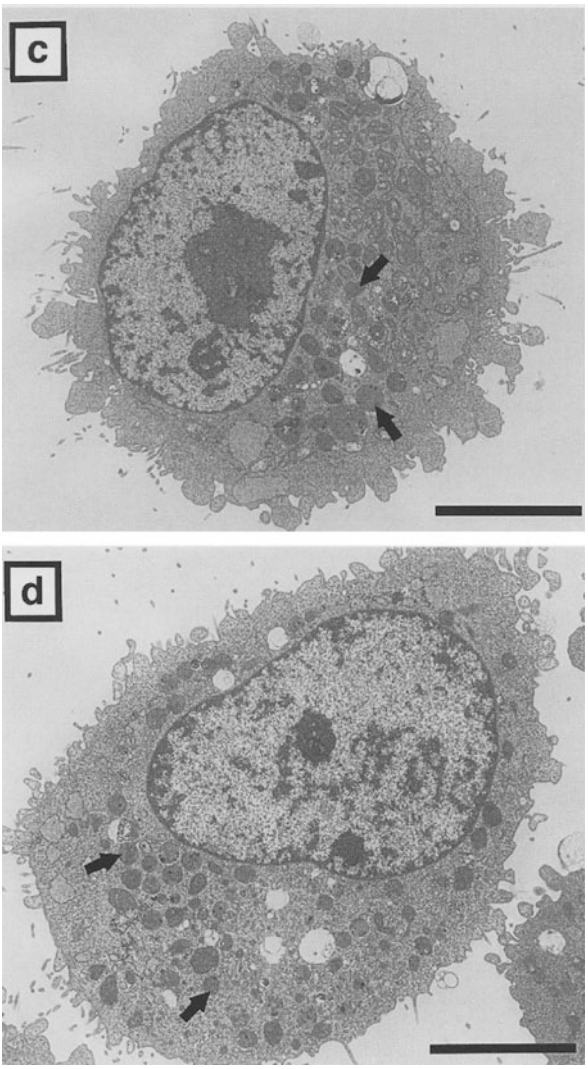

Fig. 2c–d.

cells to acquire resistance to cytotoxic substances, such as pathogenic micro-organisms or their toxins.

The finding that mutant cells selected during persistent reovirus infection do not support proteolytic disassembly of viral outer-capsid proteins led to the suggestion that mutant viruses are altered in their requirement for acid-dependent proteolysis to facilitate entry (DERMODY et al. 1993). This is indeed the case. In contrast to wt viruses, PI viruses grow well in L cells treated with ammonium

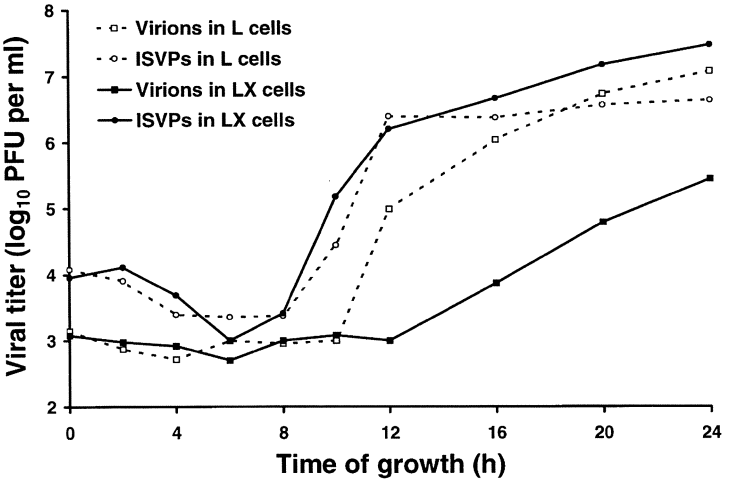

Fig. 3. Growth of wt virions and ISVPs in parental L cells and cured LX cells. Monolayers of cells (5×10^5 cells) were infected with either virions or ISVPs of wt T3D at an MOI of 2 PFU per cell. After a 1-h adsorption period the inoculum was removed, fresh medium was added, and the cells were incubated at 37°C for the times shown. 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 titers for two independent experiments. (From DERMODY et al. 1993)

chloride (DERMODY et al. 1993; WETZEL et al. 1997a,b) (Fig. 4a), which suggests that mutant cells that do not fully support virion-to-ISVP processing select mutant viruses that tolerate higher pH during steps to complete viral entry. PI viruses are also capable of efficient growth in L cells treated with E64 (BAER and DERMODY 1997), an inhibitor of cysteine proteases such as those present in the endocytic compartment (BARRETT et al. 1982) (Fig. 4b). As with ammonium chloride, E64 blocks infection by virions but not ISVPs (BAER and DERMODY 1997). Thus PI viruses are altered in their requirements for both acidification and proteolysis to facilitate entry into cells.

The reovirus σ3 and μ1/μ1C proteins are major components of the viral outer capsid (SMITH et al. 1969; DRYDEN et al. 1993), and both proteins are cleaved during conversion of virions to ISVPs (CHANG and ZWEERINK 1971; SILVERSTEIN et al. 1972; STURZENBECKER et al. 1987). Since PI viruses are capable of growth in the presence of the disassembly inhibitors ammonium chloride and E64, it was reasoned that PI virus virions undergo virion-to-ISVP processing more efficiently than virions of wt virus. This hypothesis was tested in a single study in which 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 (WETZEL et al. 1997b). Proteolysis of PI virus outer-capsid proteins σ3 and μ1C occurred with faster kinetics than proteolysis of wt virus outer-capsid proteins (Fig. 5). These results provide strong evidence that increased efficiency of proteolysis of the viral outer capsid is important for growth of reovirus in persistently infected cultures.

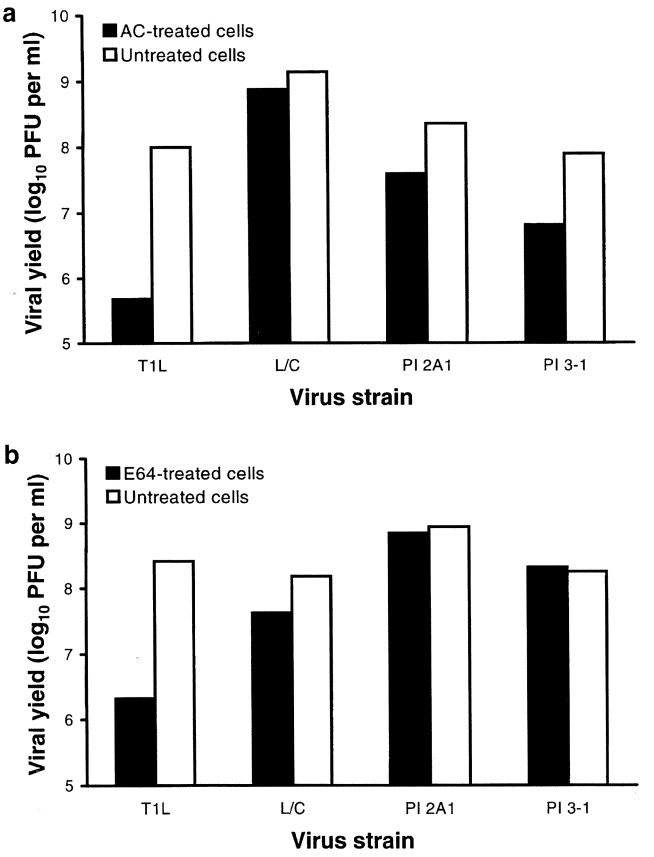

Fig. 4. Growth of wt and PI viruses in the presence and absence of (**a**) ammonium chloride (AC) and (**b**) E64. Monolayers of L cells (5×10^5cells) were infected with either wt T1L or the PI viruses shown at an MOI of 2 PFU per cell. After a 1-h adsorption period the inoculum was removed, fresh medium was added (with or without 10 mM AC or 100 μM E64), and the cells were incubated at 37°C for 10 millimolar AC 24h. 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 yields 100 micromolar E64 for two independent experiments. (Adapted from WETZEL et al. 1997b; BAER and DERMODY 1997)

5 A Model of Persistent Reovirus Infection of Cultured Cells

Studies described thus far make it possible to propose a general model for the establishment and maintenance of persistent reovirus infection (Fig. 6). Establishment of persistent infection appears to be associated with attenuation of viral cytopathicity, and depending on the cell type several mechanisms can facilitate this attenuation. These include viral passage at high multiplicity of infection (L cells) (AHMED and GRAHAM 1977; AHMED and FIELDS 1982; BROWN et al. 1983; DERMODY et al. 1993), resistance to virus-induced inhibition of cellular protein synthesis

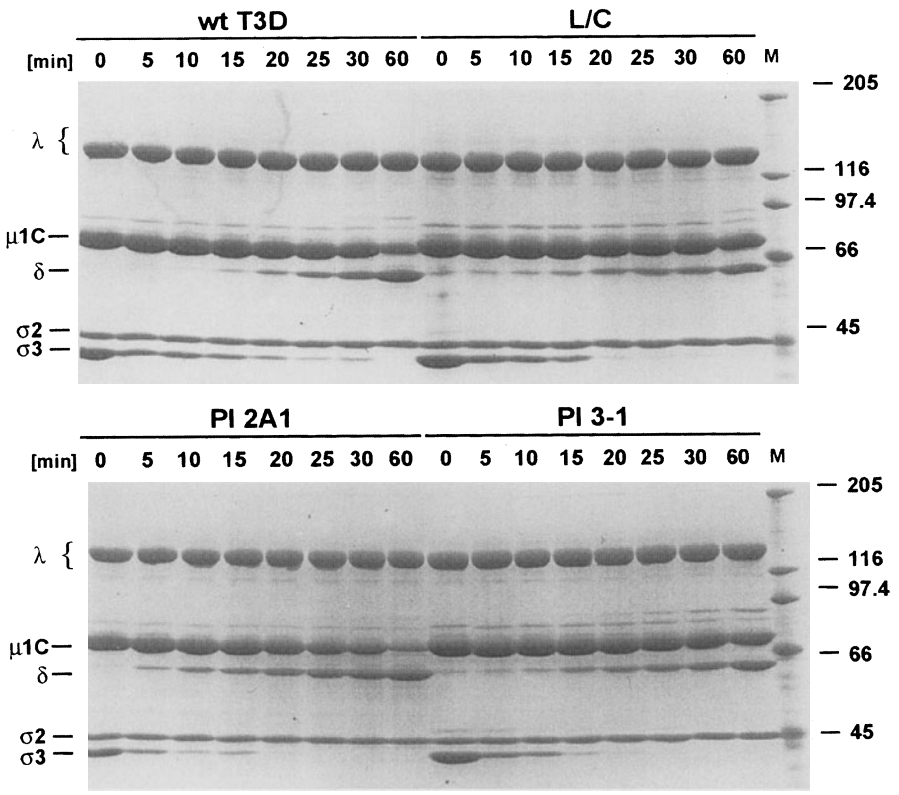

Fig. 5. Electrophoretic analysis of viral structural proteins of wt and PI virus virions during treatment with chymotrypsin to generate ISVPs. Purified virions of wt T3D and PI viruses L/C, PI 2A1, and PI 3-1 at a concentration of 8×10^{12} particles per milliliter were treated with chymotrypsin at 10°C for the times shown. Equal volumes of samples (75μl) were loaded into wells of 5%–15% polyacrylamide gradient gels. After electrophoresis gels were stained with Coomassie blue. Times (minutes) of chymotrypsin treatment are shown at the top of each gel. Viral proteins are labeled, and molecular-weight markers (in kilodaltons) appear in the lanes labeled M. (From WETZEL et al. 1997b)

(SC1 cells) (DANIS et al. 1993), and diminished capacity to support viral disassembly [L cells treated with ammonium chloride (CANNING and FIELDS 1983) and MEL cells (WETZEL et al. 1997a)]. Each of these mechanisms would result in a diminution of the effective viral inoculum, which would limit productive infection to a minority population of cells during the initial rounds of viral replication.

Cells manifesting moderate levels of resistance to viral replication would be spared and become the source for selection of an increasingly resistant cell population capable of surviving increasing viral titers. For cells with low levels of resistance to viral replication, cell crises would be expected during this period of persistent infection, leaving only those cells that support greatly reduced viral replication. During the maintenance phase of persistent infection this model predicts that mutant viruses exhibiting an augmented capacity to infect the resistant

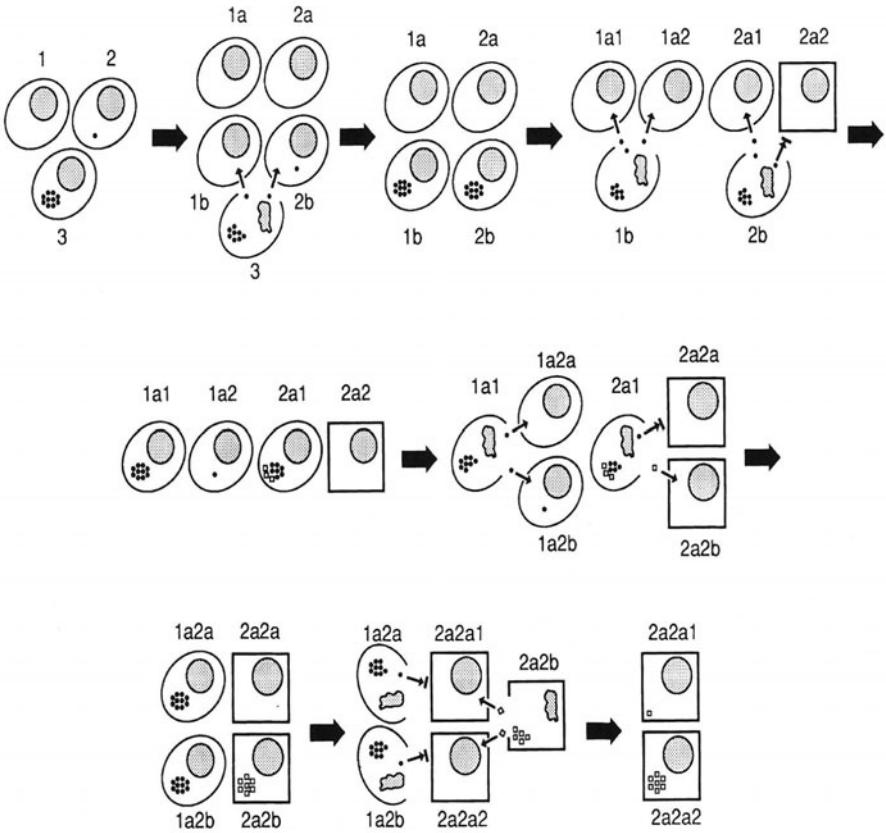

Fig. 6. Virus-cell coevolution during the maintenance of persistent reovirus infection. Cells and their progeny (large ovals and squares) and viruses and their progeny (small circles and squares) in a persistently infected culture are shown. According to this model, the culture exhibits inefficient vertical transmission of virus between cells because more severely infected cells undergo lysis (e.g., cell 3) and less severely infected cells (e.g., cell 2) undergo cell division and generate daughter cells (e.g., cells 2a and 2b) that remain susceptible to viral infection by horizontal transmission. Because of ongoing reinfection and lysis of cells within the culture, mutant cells more resistant to reinfection are selected (e.g., cell 2a2). In this model mutant viruses that are more efficient than wild-type viruses at infecting the resistant cells are selected subsequently (e.g., virus infecting cell 2a2b). In order for the persistent infection to be maintained mutant cells must not be fully permissive to infection by mutant viruses, and mutant viruses must retain the capacity to infect a subpopulation of mutant cells. (From Dermody et al. 1993)

cells would be selected. However, for these persistent infections to survive, an equilibrium between viral cytopathicity and cellular resistance must be reached in which ongoing viral replication is not sufficient to completely lyse the culture. Thus these persistent infections also can be termed chronic infections in which lysis is restricted to a subset of cells. For persistent reovirus infections of L cells and MEL cells this equilibrium rests at an early step in the viral replication cycle since these persistent infections select mutations in both cells and viruses that affect viral entry. It is possible that mutations affecting other aspects of reovirus replication are

selected during persistent infections of other cell types. In this regard persistent reovirus infections of 3T3 cells are associated with decreased expression of epidermal growth factor receptors and increased expression of insulin receptors (VERDIN et al. 1986). Additionally, persistent infections of MDCK cells are associated with decreased expression of epidermal growth factor receptors and decreased capacity to form tight junctions (MONTGOMERY et al. 1991). However, the relationship of these findings to mechanisms that serve to propagate persistent infections of 3T3 cells and MDCK cells is not known.

An important conclusion from studies of persistent reovirus infections of L cells and MEL cells is that maintenance of persistent infection selects viruses with enhanced cytopathicity. This finding is in conflict with some models of persistent infection which hold that viruses with attenuated cytopathicity are required for maintenance of persistent infection. By virtue of mutations that accelerate their disassembly PI viruses selected during persistent infections of L cells and MEL cells produce larger plaques and grow to higher titers than wt viruses (J.D. WETZEL, and T.S. DERMODY, unpublished observations). Thus mutant viruses selected by growth in cells containing blocks to viral infection have an enhanced capacity to infect parental cells. It is unlikely that such virus-cell coevolution occurs in a single infected host; however, virus-host coevolution analogous to persistent reovirus infection of cultured cells is a general feature of pathogen-host interactions in populations (FENNER and KERR 1994).

6 Viral Mutations Selected During Persistent Reovirus Infection

Recent work has focused on the identification of viral mutations that confer phenotypes selected during persistent reovirus infection with the goal of determining how these mutations lead to alterations in viral entry. These studies have been greatly facilitated by reassortant genetics in which mutations responsible for entry-enhancing phenotypes can be ascribed to specific viral genes. This approach has allowed mutations important for phenotypes required to maintain persistent infection to be distinguished from irrelevant mutations arising during prolonged viral passage in cell culture. In comparison to wt viruses, PI viruses produce significantly greater yields in mutant cells cured of persistent infection (AHMED et al. 1981; KAUFFMAN et al. 1983; DERMODY et al. 1993; WILSON et al. 1996; WETZEL et al. 1997a) and in cells treated with either ammonium chloride (DERMODY et al. 1993; WETZEL et al. 1997a,b) or E64 (BAER and DERMODY 1997).

Each of these phenotypes has been mapped genetically using reassortant viruses isolated from crosses of wt strain T1L and three independent PI viruses (Table 1). Mutations in PI viruses that confer growth in cured cells segregate with either the S1 or S4 gene segments, depending on the PI virus studied (KAUFFMAN et al. 1983; WILSON et al. 1996). Similarly, mutations that confer growth in the presence of ammonium chloride segregate with either the S1 or S4 genes (WETZEL

Table 1. Viral genes that determine growth of PI viruses in mutant cells and in cells treated with either ammonium chloride or protease inhibitor E64[a]

PI virus strain	Viral genes that segregate with growth in		
	Mutant cells	AC-treated cells	E64-treated cells
L/C	S1	S1	S4
PI 2A1	S4	S4	S4
PI 3–1	S1	S4	S4

[a] PI × wt reassortant viruses isolated from three independent crosses were tested for growth in mutant cells selected during persistent infection [T1L × L/C reassortants (KAUFFMAN et al. 1983), T1L × PI 2A1 and T1L × PI 3–1 reassortants (WILSON et al. 1996)], in the presence of ammonium chloride (AC) (WETZEL et al. 1997b), and in the presence of E64 (BAER and DERMODY 1997). Genes derived from the PI virus parent that segregate with mutant viral phenotypes are shown.

et al. 1997b). These findings are provocative and suggest that acid-dependent disassembly events during conversion of virions to ISVPs involve both viral attachment protein σ1 and outer-capsid protein σ3. In contrast, mutations that confer growth in the presence of E64 map exclusively to the S4 gene segment (BAER and DERMODY 1997), which suggests that viral susceptibility to proteolytic action is determined by the σ3 protein alone.

Sequence analysis of genes that segregate with phenotypes selected during persistent infection has revealed insight into mechanisms of entry-enhancing mutations in PI viruses. The S1 gene nucleotide sequences of seven PI viruses isolated from independent persistently infected L-cell cultures have been determined (WILSON et al. 1996). The S1 sequences of these viruses contain from one to three mutations, and with a single exception each mutation results in a change in the deduced amino acid sequence of σ1 protein (Fig. 7). The capacity of the PI virus having a wt σ1 protein to efficiently infect cured cells segregates with the S4 gene and, as would be predicted, not with the S1 gene (WILSON et al. 1996). Mutations in the σ1 proteins of several PI viruses are contained in a region of σ1 important for stability of σ1 oligomers (LEONE et al. 1991b). An oligomeric form of σ1 protein of wt T3D can be detected in sodium dodecyl sulfate polyacrylamide gels by increasing the pH of the sample buffer (BASSEL-DUBY et al. 1987). Using sample buffer conditions favoring migration of σ1 oligomers, mutations in PI virus σ1 proteins were found to decrease stability of σ1 oligomers (WILSON et al. 1996) (Fig. 8). Alterations in stability of σ1 oligomers might affect conformational changes in σ1 that occur during reovirus entry. Such a conformational change might target the virus-receptor complex to an endocytic compartment where proteolysis of the outer capsid occurs or facilitate proteolysis of outer-capsid proteins by endocytic proteases. Alternatively, mutations affecting stability of σ1 oligomers might alter 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 likely augment viral growth in cells manifesting blocks to viral disassembly, such as those selected during persistent reovirus infection.

The observation that oligomers of PI virus σ1 protein are less stable than wt σ1 at increased pH (WILSON et al. 1996) suggests that a conformational change in

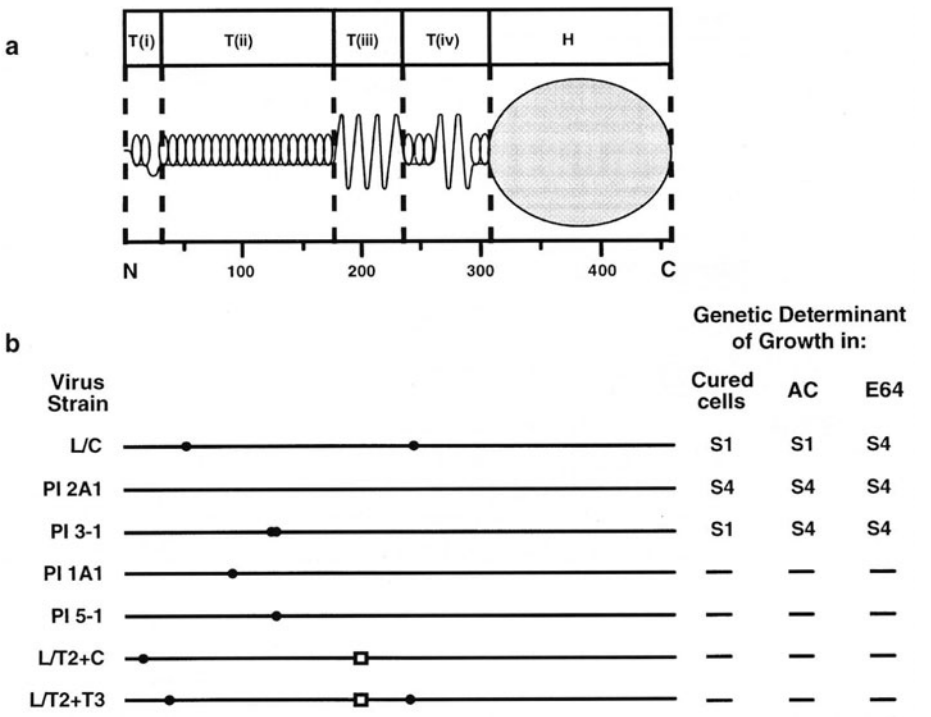

Fig. 7a,b. A structural model of the reovirus σ1 protein and location of mutations in the deduced σ1 amino acid sequences of seven PI viruses. **a** Morphological regions of σ1 (tail, *T*; head, *H*) defined by analysis of electron-microscopic images of purified σ1 (FRASER et al. 1990). The model of σ1 structure is based on analysis of deduced σ1 amino acid sequences of prototype strains of the three reovirus serotypes (NIBERT et al. 1990). 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 σ1 sequence are shown. **b** Mutations in σ1 proteins of PI viruses. *Closed circles*, sites of point mutations; *open squares*, sites of deletions. Viral genes that segregate with growth in cured cells (KAUFFMAN et al. 1983; WILSON et al. 1996), ammonium chloride (*AC*)-treated cells (WETZEL et al. 1997b), or E64-treated cells (BAER and DERMODY 1997) are indicated for PI virus strains L/C, PI 2A1, and PI 3-1; –, genetic analysis not performed. (Adapted from WILSON et al. 1996)

σ1 during viral disassembly (FURLONG et al. 1988; DRYDEN et al. 1993; NIBERT et al. 1995) is acid dependent. This contention is also supported by genetic linkage of the S1 gene and the capacity of PI virus L/C to grow in the presence of ammonium chloride (WETZEL et al. 1997b). Acid-dependent conformational changes in viral attachment proteins during disassembly have been reported for several viruses, including influenza virus (BULLOUGH et al. 1994), Semliki Forest virus (KIELIAN and HELENIUS 1985; WAHLBERG et al. 1992), and tick-borne encephalitis virus (ALLISON et al. 1995). Furthermore, pH-sensitive events involving viral attachment proteins have been shown to be altered in some types of persistent infections, including those caused by the coronavirus, mouse hepatitis virus (GALLAGHER et al. 1991).

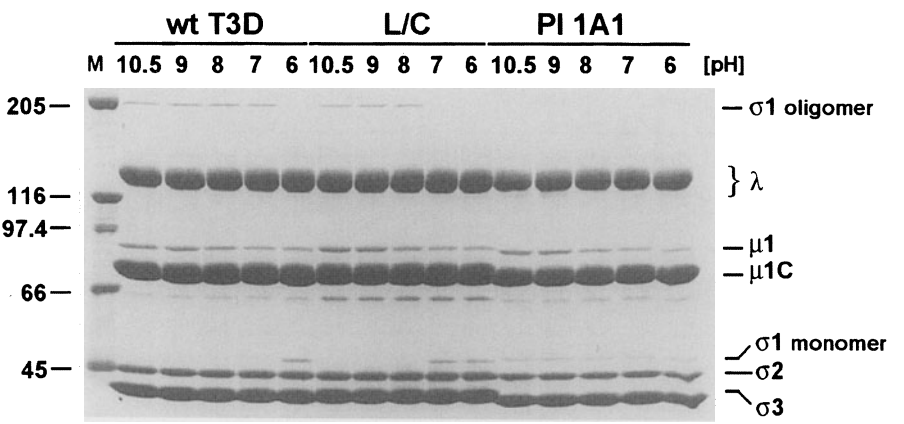

Fig. 8. Effect of pH on electrophoretic mobility of σ1 protein. Purified virions (2×10^{11} particles) of wt T3D and PI viruses L/C and PI 1A1 were incubated in sample buffer adjusted to the pH values shown prior to electrophoresis in a 5%–15% polyacrylamide gradient gel. After electrophoresis the gel was stained with Coomassie blue. Viral proteins are labeled, and molecular weight markers (in kilodaltons) appear in the lane labeled M. Oligomer and monomer bands of σ1 are indicated. (Adapted from WILSON et al. 1996)

The S4 gene nucleotide sequences of the same seven PI viruses have also been determined (WETZEL et al. 1997b). The S4 sequences of these PI viruses contain from one to four mutations, and with a single exception each mutation results in a substitution in the deduced amino acid sequence of σ3 protein (Fig. 9). Three regions of σ3 appear to be targets for mutations in the PI viruses studied: amino acids 86–145, 218–232, and 354. Six of the seven PI viruses studied contain a tyrosine to histidine substitution at residue 354, and in the case of PI virus 3-1 σ3 protein, this substitution is the only mutation observed. Since the S4 gene segregates exclusively with the growth of T1L × PI 3-1 reassortants in cells treated with either ammonium chloride (WETZEL et al. 1997b) or E64 (BAER and DERMODY 1997), it appears that a tyrosine to histidine mutation at amino acid 354 determines susceptibility of the σ3 protein to acid-dependent proteolysis. A region of σ3 adjacent to amino acid 220 is sensitive to a variety of proteases (SCHIFF et al. 1988; MILLER and SAMUEL 1992), and this region of the protein is postulated to be cleaved by endocytic proteases during viral entry (SHEPARD et al. 1995). It is possible that the tyrosine to histidine mutation at amino acid 354 alters the conformation of the σ3 cleavage site and enhances susceptibility of σ3 to proteolysis by E64-sensitive proteases. Alternatively, this mutation might alter interactions between σ3 and another outer-capsid protein such that the σ3 cleavage site is indirectly rendered more accessible to proteolysis. In support of this idea, it has been shown that interactions between σ3 and μ1 result in a conformational change in σ3 that increases its susceptibility to cleavage (SHEPARD et al. 1995). Another possibility is that the tyrosine to histidine mutation at amino acid 354 allows σ3 to be cleaved by acid-independent proteases that are not inhibited by E64.

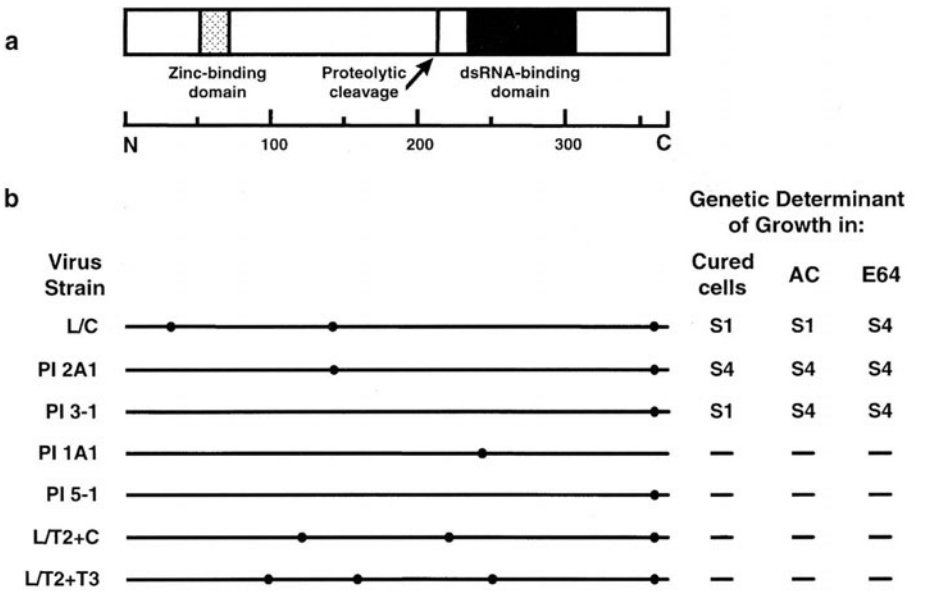

Fig. 9a,b. Location of mutations in deduced σ3 amino acid sequences of seven PI viruses. **a** Functional domains of σ3 protein (SCHIFF et al. 1988; MILLER and SAMUEL 1992). *Arrow*, a site cleaved by staphylococcal V8 protease (SCHIFF et al. 1988). Amino acid positions in σ3 sequence are shown. **b** Mutations in σ3 proteins of PI viruses. Viral genes that segregate with growth in cured cells (KAUFFMAN et al. 1983; WILSON et al. 1996), ammonium chloride (*AC*)-treated cells (WETZEL et al. 1997b), or E64-treated cells (BAER and DERMODY 1997) are indicated for PI virus strains L/C, PI 2A1, and PI 3-1; –, genetic analysis not performed. (Adapted from WETZEL et al. 1997b)

7 A Model of Reovirus Entry Derived from Studies of PI Reoviruses

The identification of viral genes that segregate with PI virus growth in cells treated with either ammonium chloride or E64 has led to the suggestion that acidification and proteolysis mediate different events in reovirus disassembly. Growth of T1L × L/C reassortants in the presence of ammonium chloride segregates with the S1 gene (WETZEL et al. 1997b), whereas growth of T1L × L/C reassortants in the presence of E64 segregates with the S4 gene (BAER and DERMODY 1997). Thus in the case of L/C, mutations in σ1 affect acid-dependent disassembly steps, and mutations in σ3 affect protease-dependent disassembly steps. The temporal relationship between these processes was tested by adding either ammonium chloride or E64d, a membrane permeable form of E64, at various times after viral adsorption (BAER and DERMODY 1997). Reovirus growth was found to be susceptible to complete blockade by both ammonium chloride and E64 only up to 30min after viral adsorption; thereafter, susceptibility to both inhibitors decreased logarithmically for an additional 30 min. At times of addition greater than 60min after ad-

sorption, neither ammonium chloride nor E64 had a significant effect on reovirus growth. Therefore these results suggest that acid-dependent and protease-dependent events are independent but temporally associated steps in reovirus disassembly and likely occur within the same cellular compartment.

Studies of reovirus mutants selected during persistent infection have identified viral structural proteins that mediate requirements for acidification and proteolysis during viral entry. Additionally, these studies have established the molecular basis for viral resistance to inhibitors of virion-to-ISVP disassembly. Based on this work, current models of reovirus entry can be revised to incorporate independent roles of acidification and proteolysis during disassembly of reovirus virions (Fig. 10). Reovirus disassembly is likely initiated by acid-dependent processes involving the σ1 and σ3 proteins, which are followed rapidly by proteolysis of σ3. These events are in turn followed by proteolysis of μ1/μ1C to yield the fully processed ISVP, which is capable of membrane penetration. Studies of PI reoviruses have identified the σ1 and σ3 proteins as critical targets for mutations that enhance reovirus entry. It is noteworthy that mutations in either protein facilitate growth in cured cells and resistance to ammonium chloride. These observations suggest that the σ1 and σ3 proteins interact to facilitate disassembly of reovirus virions, perhaps by mediating acid-dependent conformational changes required for subsequent proteolysis of the viral outer capsid.

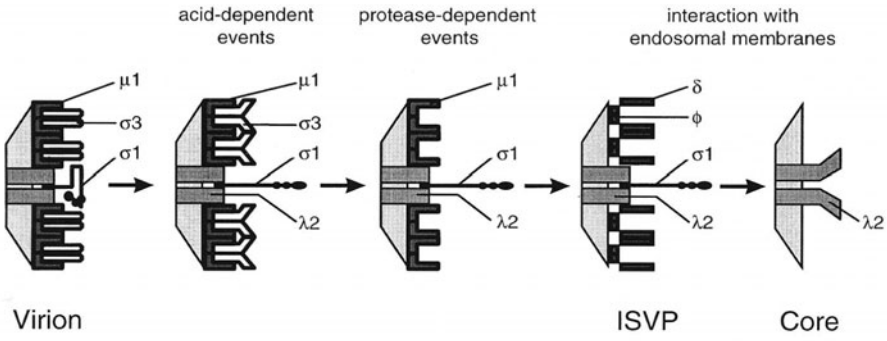

Fig. 10. A model of the disassembly of reovirus virions derived from studies of PI reoviruses. After reovirus is taken into cells by receptor-mediated endocytosis, disassembly of reovirus virions occurs in cellular endosomes by ordered changes in the viral outer capsid. Shown here are disassembly events affecting a single viral vertex. First, acid-dependent conformational changes occur in viral attachment protein σ1 and outer-capsid protein σ3. Second, the σ3 protein is degraded by endocytic proteases and lost from virions. Third, outer-capsid protein μ1/μ1C is cleaved to form μ1δ/δ and ϕ, yielding the fully processed ISVP, which is capable of interacting with endosomal membranes. The transcriptionally active viral core does not contain outer-capsid proteins and demonstrates extensive conformational changes in core-spike protein λ2. (Dryden et al. 1993)

8 Pathogenesis of PI Reoviruses

The finding that PI reoviruses are altered in viral entry led to an examination of whether these viruses are altered in virulence (MORRISON et al. 1993). Newborn NIH-Swiss mice were inoculated intracranially with 16 PI viruses, and LD_{50} values were determined for each strain. Twelve of these PI viruses had LD_{50} values identical to that of wt T3D, and four were attenuated. Of mice surviving inoculation with PI viruses at doses corresponding to the LD_{50}, 38% had detectable virus in brain tissue 25 days following infection, and of these one-half had titers greater than 1×10^5PFU. In contrast, only 16% of mice inoculated with T3D had detectable viral titer in brain tissue on day 25, and no titer was greater than 10^5PFU. Tropism of PI virus within the brain resembled that of wt virus, and the distribution of PI virus antigen in brain tissue did not change over time. By 50 days after intracranial inoculation with either wt or PI virus none of the surviving mice had detectable viral titer in brain tissue. These observations suggest that entry-enhancing mutations in PI viruses do not significantly alter viral virulence, but they do lead to prolonged viral replication in vivo.

Although reoviruses do not establish persistent infections of immunocompetent mice, they can cause persistent infections of severe combined immunodeficiency (SCID) mice (HALLER et al. 1995). Adult SCID mice inoculated intraperitoneally with wt T3D were found to survive for periods of 100 days or longer. Surviving animals harbored virus in a variety of tissues, including brain, liver, and spleen, and some of these viruses were adapted to enhanced growth in organs from which they were isolated. None of the organ-specific variants could grow in the presence of ammonium chloride, which suggests that mutations affecting acid-dependent entry steps are not selected during persistent infections in vivo. Nonetheless, the general principle of selection of viral variants dependent on the host cell is applicable to this model of persistent infection, and it is likely that analysis of variants adapted to growth in particular host tissues will reveal important new information about cell-specific factors required for efficient viral replication.

9 Future Prospects

Studies of persistent reovirus infections of cultured cells show that the cytolytic potential of mutant viruses is not diminished and that mutations in both viruses and cells affect early steps in reovirus replication involving acid-dependent viral disassembly in cellular endosomes (DERMODY et al. 1993; WETZEL et al. 1997a). These mutant viruses and cells represent a natural perturbation of the entry process and offer a unique opportunity to define mechanisms by which reovirus enters cells. Important directions for future research include a precise determination of the

cellular mutations that lead to selection of mutant viruses altered in viral entry and characterization of the molecular and structural basis of the disassembly process. Since basic mechanisms of cell entry are not well understood for most nonenveloped viruses, studies of the interplay between viral and cellular factors required to effect reovirus entry should contribute significantly to this field and illuminate new targets for therapeutic intervention in diseases caused by viruses that enter cells by receptor-mediated endocytosis. Moreover, ongoing studies of adaptive mechanisms used by viruses to overcome host-cell barriers to infection will reveal critical balance points in viral evolution and highlight strategies used by viruses to infect naive host populations.

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