JOURNAL OF VIROLOGY, July 1997, p. 4921–4928 Vol. 71, No. 7
0022-538X/97/$04.00+0

Mutations in Reovirus Outer-Capsid Protein σ3 Selected during Persistent Infections of L Cells Confer Resistance to Protease Inhibitor E64

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Received 11 December 1996/Accepted 1 April 1997

Mutations selected in reoviruses isolated from persistently infected cultures (PI viruses) affect viral entry into cells. Unlike wild-type (wt) viruses, PI viruses can grow in the presence of ammonium chloride, a weak base that blocks acid-dependent proteolysis of viral outer-capsid proteins in cellular endosomes during viral entry. In this study, we show that E64, an inhibitor of cysteine proteases such as those present in the endocytic compartment, blocks growth of wt reovirus by inhibiting viral disassembly. To determine whether PI viruses can grow in the presence of an inhibitor of endocytic proteases, we compared yields of wt and PI viruses in cells treated with E64. Prototype PI viruses L/C, PI 2A1, and PI 3-1 produced substantially greater yields than wt viruses type 1 Lang (T1L) and type 3 Dearing (T3D) in E64-treated cells. To identify viral genes that segregate with growth of PI viruses in the presence of E64, we tested reassortant viruses isolated from independent crosses of T1L and each of the prototype PI viruses for growth in cells treated with E64. Growth of reassortant viruses in the presence of E64 segregated exclusively with the S4 gene, which encodes viral outer-capsid protein σ3. These results suggest that mutations in σ3 protein selected during persistent infection alter its susceptibility to cleavage during viral disassembly. To determine the temporal relationship of acid-dependent and protease-dependent steps in reovirus disassembly, cells were infected with wt strain T1L or T3D, and medium containing either ammonium chloride or E64d, a membrane-permeable form of E64, was added at various times after adsorption. Susceptibility to inhibition by both ammonium chloride and E64 was abolished when either inhibitor was added at times greater than 60 min after adsorption. These findings indicate that acid-dependent and protease-dependent disassembly events occur with similar kinetics early in reovirus replication, which suggests that these events take place within the same compartment of the endocytic pathway.

Reoviruses are nonenveloped viruses that enter cells by receptor-mediated endocytosis (8, 9, 30, 35). Within late endosomes or lysosomes, viral outer-capsid proteins σ3 and μ1/μ1C are subject to proteolysis by cellular proteases, resulting in the generation of infectious subvirion particles (ISVPs) (9, 12, 33, 35). 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 reference 29). Treatment of cells with the weak base ammonium chloride (14, 35) or inhibitors of the vacuolar proton ATPase, such as bafilomycin or concanamycin A (22), blocks infection by virions but not by ISVPs. These findings suggest that proteolysis of σ3 and μ1/μ1C is an acid-dependent process. The vacuolar proteases that mediate cleavage of reovirus outer-capsid proteins have not been identified; however, it is likely that cysteine proteases present in the endocytic compartment, such as cathepsins B, H, and L (reviewed in reference 7), are involved in disassembly of reovirus virions.

During persistent reovirus infection of cultured cells, mutant viruses that have alterations in viral entry are selected (14, 39). In contrast to wild-type (wt) viruses, viral isolates from persistently infected cultures (PI viruses) can grow in cells treated with ammonium chloride (14, 39). These results suggest that mutant viruses have altered requirements for decreased pH to complete steps in entry leading to the generation of ISVPs. Analysis of reassortant viruses containing mixtures of gene segments from wt and PI viruses demonstrated that mutations in PI viruses that confer growth in ammonium chloride-treated cells segregate with either the S1 or S4 gene segments, depending on the PI virus studied (40). These results suggest that both viral attachment protein σ1, which is encoded by the S1 gene (23, 26), and outer-capsid protein σ3, which is encoded by the S4 gene (23, 26), serve as targets for acid-dependent disassembly events during conversion of virions to ISVPs. During chymotrypsin treatment of virions to generate ISVPs in vitro, cleavage of PI virus outer-capsid proteins occurs with faster kinetics than cleavage of wt outer-capsid proteins (40). However, it is not known whether mutations in PI viruses alter susceptibility of the viral outer capsid to proteolysis during viral entry into cells.

In this study, we used E64, an inhibitor of cysteine proteases such as those present in the endocytic compartment (3), to directly test whether protease susceptibility of PI virus outer-capsid proteins is altered during reovirus infection of cultured cells. We determined whether wt and PI viruses differ in the capacity to grow in cells treated with E64, and we used reassortant genetics to identify viral genes that segregate with PI virus growth in the presence of E64. In addition, we compared kinetics of inhibition of viral growth by ammonium chloride and E64. The results suggest that acidification and proteolysis mediate independent but temporally linked events in the disassembly of reovirus virions during viral entry.

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MATERIALS AND METHODS

Cells and viruses. Murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented to contain 5% fetal bovine serum (Intergen, Purchase, N.Y.), 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin per ml (Irvine). Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) are laboratory stocks. PI virus L/C was isolated from a persistently infected L-cell culture established with T3D temperature-sensitive mutant *ts*C447, which had been passaged serially at high multiplicity of infection (MOI) (1). PI 2A1 and PI 3-1 were isolated from persistently infected cultures established with independent high-passage stocks of T3D (14). T1L × L/C (19), T1L × PI 2A1 (41), and T1L × PI 3-1 (41) reassortant viruses were isolated previously. Purified virion preparations were made using second-passage L-cell lysate stocks of twice-plaque-purified reovirus as previously described (16). Purified virions containing ^{35}S-labeled proteins were obtained by adding Tran^{35}S-label methionine (ICN Biochemicals, Costa Mesa, Calif.) to cell suspensions at the initiation of infection (~1.25 μCi/ml).

Growth of reovirus in the presence and absence of E64. Monolayers of L cells (5×10^5 cells) in 24-well plates (Costar, Cambridge, Mass.) were preincubated for 1 h in medium supplemented to contain from 0 to 200 μM E64 (Sigma Chemical Co., St. Louis, Mo.). The medium was removed, and cells were infected with reovirus strains at an MOI of 2 PFU per cell. After a 1-h adsorption period at 4°C, the inoculum was removed, cells were washed twice with phosphate-buffered saline (PBS), and 0.5 ml of fresh medium supplemented with E64, from 0 to 200 μM, 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 (36). Independent experiments were performed with single wells of cells, which were titrated in duplicate.

Preparation of ISVPs. Purified reovirus virions at a concentration of 2×10^{12} particles per ml in virion storage buffer (150 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris [pH 7.4]) were digested with 200 μg of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine α-chymotrypsin (Sigma) per ml at 37°C for 2 h. Chymotrypsin digestions were stopped by adding phenylmethylsulfonyl fluoride (Sigma) to the treatment mixtures (1 mM, final concentration) and cooling to 0°C.

Infection of cells with radiolabeled reovirus virions. Monolayers of L cells (10^7 cells) in 75-cm^2 flasks (Costar) were preincubated for 1 h in medium supplemented to contain from 0 to 200 μM E64. The medium was removed, and cells were adsorbed with purified, ^{35}S-labeled reovirus virions at 10,000 particles per cell. After 1 h at 4°C, the inoculum was removed, cells were washed twice with PBS, and 10 ml of fresh medium supplemented with E64, from 0 to 200 μM, was added. After incubation at 37°C for defined intervals, cells were harvested by using a cell scraper and collected by centrifugation at 528 × *g* for 5 min. Cells were resuspended in 0.5 ml of lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 0.5% Nonidet P-40, 1 mM EDTA, 1 mM benzamidine [Sigma], 100 mM leupeptin [Sigma], 2.5 mM phenylmethylsulfonyl fluoride) and placed on ice for 30 min, and 4.5 ml of homogenization buffer (250 mM NaCl, 10 mM Tris [pH 7.4], 0.067% 2-mercaptoethanol) was added. Samples were sonicated for 1 min, 2.5 ml of Freon (EM Science, Gibbstown, N.J.) was added, and samples were again sonicated for 1 min. Samples were centrifuged at 9,700 × *g* for 10 min, and the aqueous fraction was placed into 14- by 89-mm centrifuge tubes (Beckman, Palo Alto, Calif.). Virus particles were pelleted by centrifugation in an SW41 rotor (Beckman) at 210,000 × *g* for 1 h.

SDS-PAGE of reovirus structural proteins. Discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed as previously described (21). Viral particles were solubilized by incubation in sample buffer (125 mM Tris, 2% 2-mercaptoethanol, 1% SDS, 0.01% bromophenol blue) at 65°C for 5 min. Samples were loaded into wells of 10% polyacrylamide gels and electrophoresed at 20-mA constant current for 12 h. Following electrophoresis, gels were fixed, dried onto filter paper (Bio-Rad Laboratories, Richmond, Calif.) under vacuum, and exposed to BioMax film (Eastman Kodak Co., Rochester, N.Y.).

Statistical analysis. The association of reovirus gene segments with growth in L cells treated with E64 was determined by using both nonparametric (Mann-Whitney [MW] test) and parametric (*t*-test) statistical techniques. Statistical analyses were performed by using the Minitab (release 8) statistical software package (Addison-Wesley, Reading, Mass.).

Kinetics of inhibition of reovirus growth mediated by ammonium chloride and E64d. Monolayers of L cells (5×10^5 cells) were infected with reovirus strains at an MOI of 2 PFU per cell. After a 1-h adsorption period at 4°C, the inoculum was removed, cells were washed twice with PBS, fresh medium was added, and cells were incubated at 37°C. At defined intervals, the medium was removed, fresh medium supplemented to contain either 20 mM ammonium chloride or 100 μM E64d (Matreya, Pleasant Gap, Pa.) (in 0.035% dimethyl sulfoxide [Sigma]) was added, and cells were incubated at 37°C for 24 h. Cells were then frozen and thawed twice, and virus in cell lysates was titrated on L-cell monolayers by plaque assay. At the concentration used, dimethyl sulfoxide alone had no effect on viral replication.

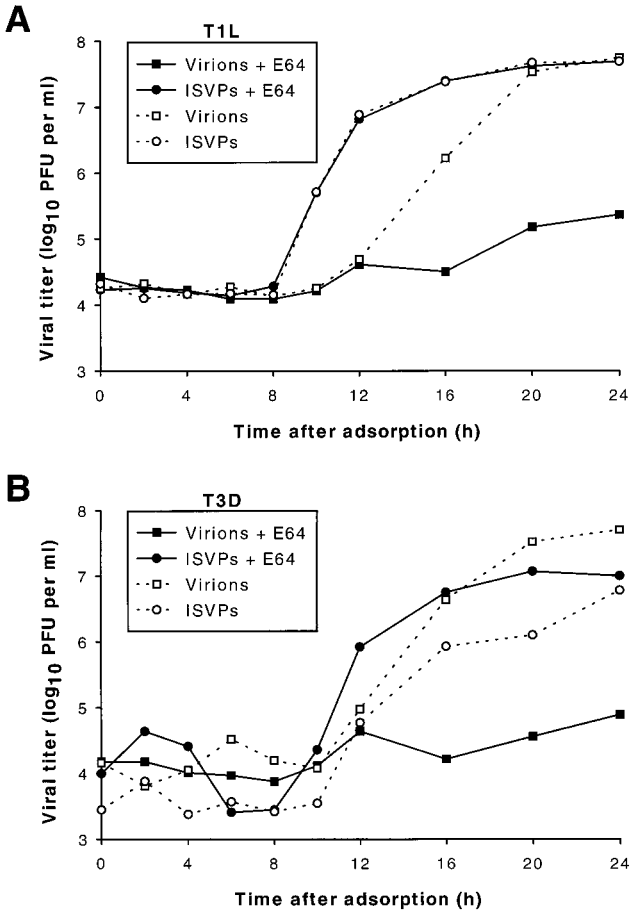

FIG. 1. Growth of virions and ISVPs of reovirus strains T1L (A) and T3D (B) in L cells maintained in the presence and absence of protease inhibitor E64. Monolayers of L cells (5×10^5 cells) were preincubated for 1 h in medium supplemented with or without 100 μM E64. The medium was removed, and cells were infected with either virions or ISVPs of each virus strain at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium with or without 100 μM E64 was added, and cells were incubated at 37°C for the times shown. Cells then were frozen and thawed twice, and virus in cell lysates was titrated on L-cell monolayers by plaque assay. The results are presented as mean viral titers for two independent experiments.

RESULTS

Effect of protease inhibitor E64 on reovirus replication. To determine the effect of an inhibitor of cysteine proteases on reovirus replication, we tested growth of wt reovirus strains T1L and T3D in L cells treated with E64. Cells were infected with virus strains at an MOI of 2 PFU per cell and incubated in either the presence or absence of 100 μM E64 (Fig. 1). After 24 h of viral growth, yields of T1L and T3D in untreated cells were approximately 100-fold greater than those in cells treated with E64. In control experiments, we did not detect cytotoxicity in cells maintained for 24 h in medium containing from 25 to 200 μM E64, as assessed by decreases in cell doubling times or trypan blue exclusion (data not shown). Therefore, these findings indicate that E64 is a potent inhibitor of reovirus replication that does not appreciably affect cell growth or viability at the concentrations tested.

To determine whether E64 inhibits reovirus growth by blocking viral disassembly, ISVPs of T1L and T3D, generated in vitro by chymotrypsin treatment of virions, were used to infect L cells in the presence and absence of E64 (Fig. 1).

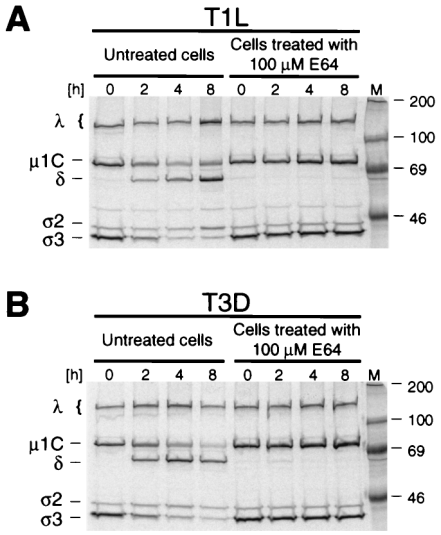

FIG. 2. Electrophoretic analysis of viral structural proteins of reovirus strains T1L (A) and T3D (B) after infection of L cells in the presence and absence of E64. Monolayers of L cells (10^7 cells) were preincubated for 1 h in medium supplemented with or without 100 μM E64. The medium was removed, and cells were adsorbed with purified ^{35}S-labeled virions of each virus strain at 10,000 particles per cell. After 1 h, the inoculum was removed, fresh medium with or without 100 μM E64 was added, and the cells were incubated at 37°C for the times shown. Cells were then lysed with lysis buffer and extracted with Freon. Viral particles contained in supernatants were pelleted by ultracentrifugation and solubilized in sample buffer. Equal volumes of samples were loaded into wells of 10% polyacrylamide gels. After electrophoresis, gels were prepared for fluorography and exposed to film. Times after adsorption are shown above each gel. Viral proteins are labeled, and positions of molecular weight markers in kilodaltons are indicated.

ISVPs generated by chymotrypsin treatment in vitro are indistinguishable from those generated in the endocytic compartment during reovirus entry into cells (9, 12, 33, 35). After conversion of T1L and T3D virions to ISVPs, both strains were capable of efficiently infecting E64-treated cells and produced yields equivalent to those of virions and ISVPs in untreated cells. Thus, ISVPs generated in vitro bypass the E64-mediated block to reovirus replication, suggesting that this block is accomplished by inhibiting viral disassembly in cellular endosomes.

To determine whether E64 specifically blocks proteolysis of the viral outer capsid during viral entry into cells, ^{35}S-labeled virions of T1L and T3D were adsorbed to L cells in the presence and absence of 100 μM E64. At various times after adsorption, viral structural proteins were resolved by SDS-PAGE and visualized by autoradiography (Fig. 2). In untreated cells infected with either T1L or T3D, we observed degradation of the σ3 protein and generation of the δ cleavage fragment of the μ1C protein within 2 h postadsorption, consistent with formation of ISVPs (9, 12, 33, 35). However, in cells treated with E64, no proteolysis of outer-capsid proteins of either virus strain was detectable for up to 8 h postadsorption. These results demonstrate that E64 acts to inhibit reovirus replication by blocking proteolytic disassembly of virions to ISVPs.

Growth of PI viruses in the presence of protease inhibitor E64. Reoviruses isolated from persistently infected cultures can grow in the presence of the disassembly inhibitor ammonium chloride (14, 39). To determine whether PI viruses also can grow in the presence of protease inhibitor E64, we compared yields of wt and PI viruses after growth in L cells treated with E64 over a range of concentrations from 25 to 200 μM (Fig. 3). For these experiments, we used prototype PI viruses L/C, PI 2A1, and PI 3-1. 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 stocks of T3D (14). Viral yields in the presence and absence of E64 were determined by plaque assay after 24 h of viral growth. To standardize for differences in viral growth, yields in E64-treated cells were divided by those in untreated L cells to calculate E64/L ratios for each virus strain. E64/L ratios of the PI viruses were greater than those of wt viruses T1L and T3D at all concentrations of E64 tested. At 200 μM E64, which was the highest concentration used, the E64/L ratios of the PI viruses were approximately 50-fold greater than those of the wt viruses. These findings indicate that reovirus mutants selected during persistent infection display a decreased susceptibility to blockade of viral replication by protease inhibitor E64.

To determine whether differences in growth of wt and PI viruses in the presence of E64 are linked to differences in viral disassembly, ^{35}S-labeled virions of wt T3D and PI 2A1 were adsorbed to L cells treated with concentrations of E64 from 25 to 200 μM. At 16 h after adsorption, viral structural proteins were resolved by SDS-PAGE and visualized by autoradiography (Fig. 4). Proteolysis of the σ3 and μ1C proteins was observed after infection of untreated cells with either T3D or PI 2A1. After infection of cells treated with E64, we observed a concentration-dependent inhibition of the proteolysis of T3D outer-capsid proteins but only a minimal effect on the proteolysis of PI 2A1 outer-capsid proteins. These findings show that PI viruses differ substantially from wt viruses in the capacity to complete disassembly steps leading to generation of ISVPs in the presence of E64.

Identification of viral genes that segregate with PI virus growth in the presence of E64. To determine mechanisms by

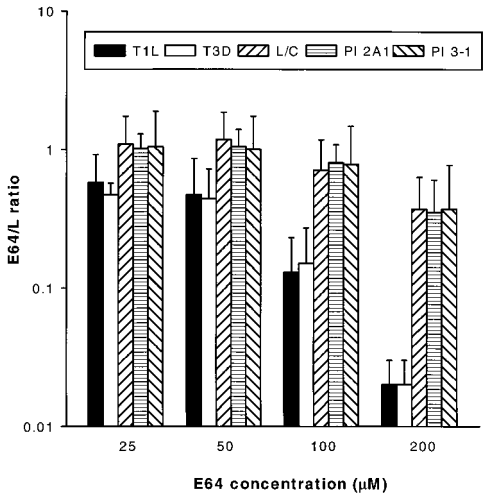

FIG. 3. Effect of E64 concentration on the growth of wt reovirus strains T1L and T3D and PI reoviruses L/C, PI 2A1, and PI 3-1. Monolayers of L cells (5×10^5 cells) were preincubated for 1 h in medium supplemented with or without E64 at the concentrations shown. The medium was removed, and cells were infected with each virus strain at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium with or without E64 was added, and the cells were incubated at 37°C for 24 h. Cells then 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 viral yield in L cells treated with E64 divided by the viral yield in untreated L cells (E64/L ratio) for four independent experiments. Error bars indicate standard deviations of the means. Viral yields (in PFU) after 24 h of growth in untreated cells were 3.0×10^8 for T1L, 2.4×10^8 for T3D, 2.3×10^8 for L/C, 4.2×10^8 for PI 2A1, and 3.2×10^8 for PI 3-1.

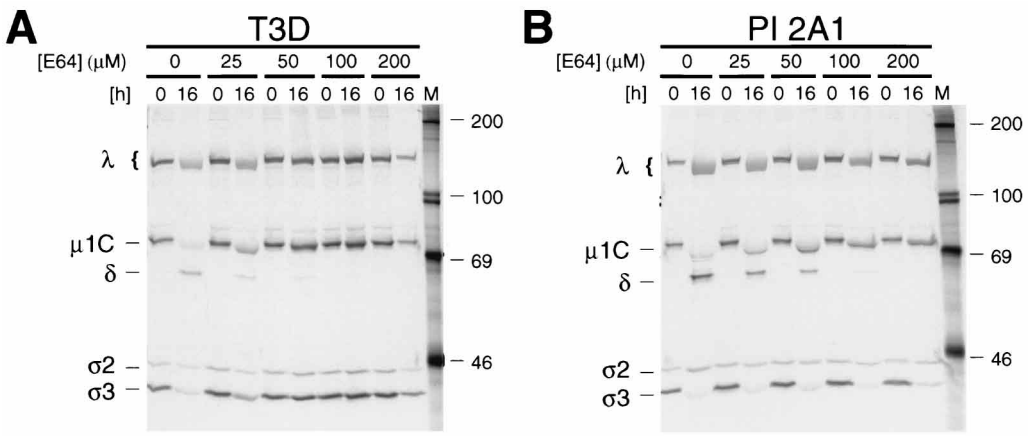

FIG. 4. Electrophoretic analysis of viral structural proteins of wt T3D (A) and PI 2A1 (B) after infection of L cells in the presence and absence of E64. Monolayers of L cells (10^7 cells) were preincubated for 1 h in medium supplemented with or without E64 at the concentrations shown. The medium was removed, and cells were adsorbed with purified ^{35}S-labeled virions of each virus strain at 10,000 particles per cell. After 1 h, the inoculum was removed, fresh medium with or without E64 was added, and the cells were incubated at 37°C for the times shown. Cells then were lysed with lysis buffer and extracted with Freon. Viral particles contained in supernatants were pelleted by ultracentrifugation and solubilized in sample buffer. Equal volumes of samples were loaded into wells of 10% polyacrylamide gels. After electrophoresis, gels were prepared for fluorography and exposed to film. The E64 concentration and times after adsorption are shown above each gel. Viral proteins are labeled, and positions of molecular weight markers in kilodaltons are indicated.

which mutations selected during persistent reovirus infection alter proteolytic susceptibility of the viral outer capsid, we used reassortant genetics to identify viral genes associated with growth of PI viruses in the presence of E64. Reassortant viruses isolated from independent crosses of wt T1L and PI viruses, L/C, PI 2A1, and PI 3-1, were tested for growth in the presence and absence of 200 μM E64. This concentration of E64 was chosen to maximize differences in growth between wt and PI viruses (Fig. 3). Viral yields in the presence and absence of E64 were determined after 24 h of viral growth, and E64/L ratios were calculated for each reassortant virus (Tables 1 to 3). In each case, reassortant viruses containing an S4 gene derived from the PI virus parent had E64/L ratios of >0.1. In sharp contrast, those with an S4 gene from T1L had E64/L ratios of <0.02, with a single exception. T1L × PI 3-1 reassortant virus LD145, which contains an S4 gene derived from T1L, had an E64/L ratio of 0.086. No other reovirus genes were associated with E64/L ratios of >0.1, which suggests that mutations in the S4 gene alone determine the capacity of PI viruses to grow better than wt viruses in the presence of E64.

To confirm the association of the S4 gene with growth of T1L × PI virus reassortants in E64-treated cells, we analyzed the results by using nonparametric and parametric statistical techniques. The results of these analyses demonstrated a significant association between growth of reassortant viruses in the presence of E64 and the S4 gene (T1L × L/C reassortants: MW test, $P = 0.03$, and t test, $P = 0.036$; T1L × PI 2A1 reassortants: MW test, $P = 0.037$, and t test, $P = 0.0078$; T1L × PI 3-1 reassortants: MW test, $P = 0.036$, and t test, $P = 0.0002$). By t test only, the L1 gene was associated with growth of T1L × PI 2A1 reassortants in cells treated with E64 ($P = 0.016$). No other viral genes were significantly associated with

TABLE 1. Growth of T1L × L/C reassortant viruses in the presence and absence of E64

Virus strain	Origin of gene segment[a]										Yield in[b]:		E64/L ratio[c]	Rank[d]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	E64-treated cells	Untreated cells		
Parental														
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	5.6×10^5	1.9×10^8	0.0029	
L/C	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	4.2×10^7	1.5×10^8	0.28	
Reassortant														
RK49	wt	PI	wt	wt	wt	PI	PI	wt	wt	PI	1.1×10^8	2.6×10^8	0.42	1
RK96	PI	PI	PI	PI	PI	PI	wt	PI	PI	PI	2.0×10^7	9.1×10^7	0.22	2
RK23	PI	wt	PI	PI	PI	PI	PI	PI	PI	PI	1.5×10^7	9.3×10^7	0.16	3
RK26	PI	PI	PI	PI	PI	PI	wt	PI	wt	PI	1.9×10^7	1.4×10^8	0.14	4
RK99	wt	PI	wt	wt	wt	PI	wt	PI	PI	wt	5.5×10^5	1.5×10^8	0.0037	5
RK87	wt	PI	wt	PI	wt	wt	PI	PI	wt	wt	3.1×10^5	1.3×10^8	0.0024	6
RK41	PI	PI	wt	wt	wt	PI	PI	PI	PI	wt	2.8×10^5	1.2×10^8	0.0023	7
RK103	PI	PI	PI	PI	PI	PI	PI	PI	wt	wt	1.6×10^5	9.9×10^7	0.0016	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 cells) 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 200 μM E64 was added, and 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. The results are presented as mean viral yields for four independent experiments.

[c] The mean viral yield in E64-treated cells was divided by that in untreated L cells to calculate an E64/L ratio for each virus strain.

[d] Reassortant viruses are ranked from highest to lowest based on their E64/L ratios.

TABLE 2. Growth of T1L × PI 2A1 reassortant viruses in the presence and absence of E64

Virus strain	Origin of gene segment[a]										Yield in[b]:		E64/L ratio[c]	Rank[d]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	E64-treated cells	Untreated cells		
Parental														
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	1.0×10^6	4.5×10^8	0.0022	
PI 2A1	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	7.0×10^8	8.7×10^8	0.80	
Reassortant														
JW35	PI	PI	PI	wt	wt	wt	PI	wt	wt	PI	3.3×10^8	4.8×10^8	0.69	1
JW222	PI	PI	wt	PI	PI	PI	wt	PI	PI	PI	2.8×10^8	5.8×10^8	0.48	2
JW106	PI	PI	PI	PI	wt	PI	PI	PI	wt	PI	2.6×10^8	5.5×10^8	0.47	3
JW22	PI	PI	PI	PI	wt	PI	wt	wt	PI	PI	9.7×10^7	2.1×10^8	0.46	4
JW33	PI	PI	PI	wt	PI	wt	wt	PI	PI	PI	1.8×10^7	1.4×10^8	0.13	5
JW101	wt	PI	wt	wt	wt	wt	wt	PI	PI	wt	3.7×10^5	1.4×10^8	0.0026	6
JW260	PI	PI	wt	PI	PI	PI	wt	PI	PI	wt	2.5×10^5	1.4×10^8	0.0018	7
JW52	wt	wt	wt	wt	wt	wt	wt	PI	PI	wt	5.5×10^5	6.8×10^8	0.00081	8

[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 cells) 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 200 μM E64 was added, and 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. The results are presented as mean viral yields for four independent experiments.
[c] The mean viral yield in E64-treated cells was divided by that in untreated L cells to calculate an E64/L ratio for each virus strain.
[d] Reassortant viruses are ranked from highest to lowest based on their E64/L ratios.

growth of T1L × PI virus reassortants in E64-treated cells by these tests ($P > 0.05$).

As a control to exclude the possibility that certain combinations of T1L and T3D genes might confer viral growth in the presence of E64, 12 reassortant viruses isolated from crosses of T1L and T3D (10, 13, 38), the wt strain from which the PI viruses were derived (1, 14), were tested for growth in E64-treated cells (Table 4). E64/L ratios of the T1L × T3D reassortant viruses were ≤0.05, and no T1L or T3D genes were associated with differences in the capacity of these reassortants to grow in the presence of E64. Therefore, mutations in the S4 gene selected during persistent infection are associated with growth of PI viruses in L cells treated with E64.

Kinetics of inhibition of reovirus replication induced by ammonium chloride and E64. Treatment of L cells with ammonium chloride inhibits reovirus replication by blocking virion-to-ISVP disassembly (14, 35). Experiments described thus far demonstrate that treatment of L cells with protease inhibitor E64 also inhibits reovirus replication by blocking conversion of virions to ISVPs. To determine whether acid- and protease-dependent steps required for reovirus entry occur with similar kinetics, virions of either T1L or T3D were adsorbed to L cells, and medium containing either 20 mM ammonium chloride or 100 μM E64 was added to cells after various times of incubation. Following addition of inhibitor-containing medium, cells were incubated for 24 h, and virus in cell lysates was titrated by plaque assay (Fig. 5). For these experiments, we used E64d, an uncharged analog of E64 that is membrane permeable and rapidly distributes to all intracellular compartments (24). Treatment of L cells with 100 μM E64d resulted in blockade of reovirus replication identical to that induced by E64 and did not produce cytotoxicity, as assessed by decreased cell doubling times or trypan blue exclusion at concentrations as high as 800 μM (data not shown).

TABLE 3. Growth of T1L × PI 3-1 reassortant viruses in the presence and absence of E64

Virus strain	Origin of gene segment[a]										Yield in[b]:		E64/L ratio[c]	Rank[d]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	E64-treated cells	Untreated cells		
Parental														
T1L	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	4.7×10^6	1.5×10^8	0.031	
PI 3-1	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	2.1×10^8	2.8×10^8	0.75	
Reassortant														
LD134	wt	wt	wt	wt	wt	PI	PI	wt	wt	PI	1.1×10^8	2.2×10^8	0.50	1
LD15	PI	PI	PI	PI	PI	PI	wt	PI	PI	PI	4.5×10^7	9.1×10^7	0.49	2
LD10	PI	PI	wt	wt	wt	PI	PI	PI	wt	PI	5.5×10^7	1.2×10^8	0.46	3
LD125	PI	PI	wt	PI	PI	PI	wt	PI	PI	PI	1.9×10^7	4.7×10^7	0.40	4
LD130	wt	PI	PI	wt	wt	PI	wt	wt	PI	PI	1.5×10^7	4.8×10^7	0.31	5
LD145	wt	wt	wt	wt	wt	wt	PI	wt	wt	wt	1.2×10^7	1.4×10^8	0.086	6
LD18	PI	PI	PI	PI	PI	PI	wt	PI	PI	wt	9.5×10^5	5.8×10^7	0.016	7.5
LD110	PI	PI	wt	PI	PI	PI	PI	PI	wt	wt	8.3×10^5	5.2×10^7	0.016	7.5

[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 cells) 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 200 μM E64 was added, and 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. The results are presented as mean viral yields for four independent experiments.
[c] The mean viral yield in E64-treated cells was divided by that in untreated L cells to calculate an E64/L ratio for each virus strain.
[d] Reassortant viruses are ranked from highest to lowest based on their E64/L ratios.

TABLE 4. Growth of T1L × T3D reassortant viruses in the presence and absence of E64

Virus strain	Origin of gene segment[a]										Yield in[b]:		E64/L ratio[c]	Rank[d]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	E64-treated cells	Untreated cells		
Parental														
T1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	1.0×10^6	2.9×10^7	0.034	
T3D	3D	3D	3D	3D	3D	3D	3D	3D	3D	3D	1.8×10^5	4.9×10^6	0.037	
Reassortant														
H9	3D	3D	1L	3D	1L	1L	3D	3D	3D	3D	3.5×10^6	7.0×10^7	0.050	1
G2	1L	3D	1L	1L	1L	1L	3D	1L	1L	1L	3.1×10^6	6.3×10^7	0.049	2
EB121	3D	3D	1L	3D	1L	3D	1L	3D	3D	3D	8.0×10^5	1.9×10^7	0.042	3
H41	3D	3D	1L	1L	1L	3D	1L	3D	3D	1L	1.4×10^6	4.1×10^7	0.034	4
KC19	1L	1L	1L	1L	3D	1L	3D	1L	3D	1L	5.8×10^6	2.2×10^8	0.026	5
KC9	3D	3D	3D	3D	3D	3D	1L	3D	3D	3D	3.0×10^5	1.2×10^7	0.025	6
EB1	1L	3D	1L	1L	3D	1L	1L	1L	3D	1L	8.6×10^5	4.2×10^7	0.020	7
1HA3	1L	1L	1L	1L	1L	1L	3D	1L	1L	1L	4.7×10^6	3.0×10^8	0.016	8
EB31	1L	1L	1L	3D	1L	1L	1L	3D	3D	1L	2.3×10^6	2.1×10^8	0.011	9
EB28	3D	3D	1L	3D	3D	3D	3D	1L	3D	3D	1.3×10^6	1.4×10^8	0.009	10
H15	1L	3D	3D	1L	3D	3D	3D	3D	3D	1L	4.6×10^5	1.1×10^8	0.004	11
EB68	1L	3D	1L	1L	3D	1L	1L	1L	3D	3D	7.3×10^5	2.6×10^8	0.003	12

[a] Parental origin of each gene segment: 1L, gene segment derived from T1L; 3D, gene segment derived from T3D.
[b] L cells (5×10^5 cells) 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 200 μM E64 was added, and 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. The results are presented as mean viral yields for two independent experiments.
[c] The mean viral yield in E64-treated cells was divided by that in untreated L cells to calculate an E64/L ratio for each virus strain.
[d] Reassortant viruses are ranked from highest to lowest based on their E64/L ratios.

Susceptibility to ammonium chloride-mediated inhibition decreased logarithmically when ammonium chloride was added from 30 to 60 min after adsorption, consistent with previous results (14, 35). Susceptibility to E64d-mediated inhibition similarly decreased when E64d was added from 30 to 60 min after adsorption. These findings indicate that acid-dependent and protease-dependent events are temporally associated during reovirus disassembly.

DISCUSSION

In this study, we show that protease inhibitor E64 is a potent inhibitor of reovirus replication that acts by blocking disassembly of virions to ISVPs. In contrast to wt viruses, PI viruses are resistant to growth inhibition mediated by E64. Using reassortant genetics, we show that growth of three independent PI viruses in E64-treated cells segregates exclusively with the S4 gene, which encodes outer-capsid protein σ3. These results provide strong evidence that viral mutations selected during persistent reovirus infection alter susceptibility of the viral outer capsid to proteolytic cleavage during viral entry.

E64 blocks conversion of virions to ISVPs during reovirus entry into cells. All viruses must possess a mechanism to deliver their nucleic acid across cell membranes. For enveloped viruses like influenza virus (11, 34), Semliki Forest virus (20, 37), and tick-borne encephalitis virus (2), acid-dependent conformational changes in viral envelope glycoproteins facilitate fusion of the viral envelope with membranes of endocytic vesicles. For nonenveloped viruses, the cell membrane must be traversed by a different mechanism. Some nonenveloped viruses, such as adenovirus (17), astrovirus (15), parvovirus (4), and reovirus (35), rely on endocytic uptake and acidification of intracellular vesicles to gain access to the cytosol. Furthermore, proteolysis of capsid components also is necessary for entry of some of these viruses, including reovirus (9, 35). In this report, we show that treatment of cells with protease inhibitor E64 specifically blocks the proteolysis of reovirus outer-capsid proteins during viral entry. This conclusion is supported by two lines of evidence. First, ISVPs, which are disassembly intermediates generated by protease treatment of virions, bypass the E64-mediated block to reovirus replication. Second, conversion of virions to ISVPs is not observed after radiolabeled reovirus virions are used to infect cells treated with E64. Thus, our studies identify E64 as a novel inhibitor of reovirus entry. Moreover, since E64 is a specific inhibitor of proteases containing active-site cysteine residues (3), our results suggest that cysteine proteases are important for cleavage of the reovirus outer capsid within the endocytic compartment.

In addition to cellular endosomes, reovirus virions are converted to ISVPs in the intestinal lumen (5, 6). Protease inhibitors aprotinin and chymostatin block reovirus infection after peroral inoculation of newborn mice, and this blockade to infection is associated with inhibition of virion-to-ISVP conversion by intestinal proteases (5). Therefore, our results of assays using a cell culture system concur with those of in vivo studies indicating that protease inhibitors block reovirus growth by preventing viral disassembly.

Mutations in σ3 confer growth in the presence of protease inhibitor E64. Our previous studies of PI reoviruses indicate that mutations in either the S1 or S4 gene selected during persistent infection determine the capacity of PI viruses to grow in cells cured of persistent infection (41) and in cells treated with ammonium chloride (40) (Table 5). We now show that mutations in the S4 gene segregate with viral growth in cells treated with protease inhibitor E64 (Table 5). The S4 gene encodes the σ3 protein (23, 26), which is degraded and lost from virions during conversion of virions to ISVPs (9, 12, 33, 35). PI viruses produce substantially greater yields than wt viruses in the presence of E64, and PI virus virions can complete disassembly steps leading to generation of ISVPs in E64-treated cells. These findings suggest that, in comparison to wt σ3 proteins, PI virus σ3 proteins have evolved increased sensitivity to E64-inhibitable proteases or have become susceptible to other proteases not inhibited by E64.

The deduced amino acid sequences of the σ3 proteins of the PI viruses studied contain a tyrosine-to-histidine mutation at amino acid 354 (40). In the case of PI 3-1 σ3 protein, histidine

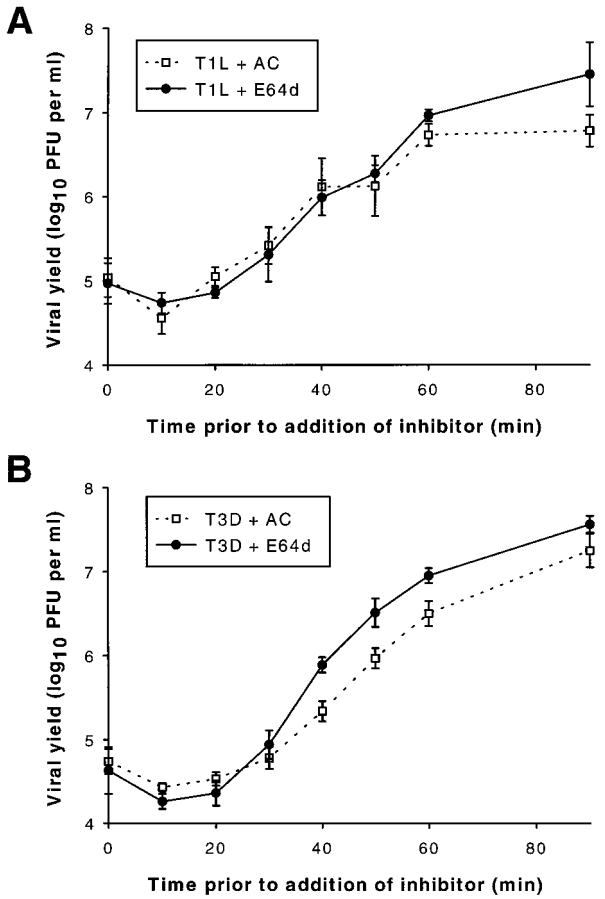

FIG. 5. Effect of time of ammonium chloride or E64d addition after virus adsorption on the growth of reovirus strains T1L (A) and T3D (B). Monolayers of L cells (5×10^5 cells) were infected with each virus strain at an MOI of 2 PFU per cell. After a 1-h adsorption period, the inoculum was removed, fresh medium was added, and the cells were incubated at 37°C. After incubation for the times shown, the medium was removed, fresh medium supplemented to contain either 20 mM ammonium chloride (AC) or 100 μM E64d was added, and cells were incubated at 37°C for 24 h. Cells then were frozen and thawed twice, and virus in cell lysates was titrated on L-cell monolayers by plaque assay. The results are presented as mean viral yields for four independent experiments. Error bars indicate standard deviations of the means.

354 is the only mutation observed. Since the S4 gene segregates exclusively with growth of T1L × PI 3-1 reassortants in E64-treated cells, these sequence data argue that a tyrosine-to-histidine mutation at amino acid 354 influences protease susceptibility of σ3. A region of σ3 adjacent to amino acid 220 is sensitive to a variety of proteases (25, 31), and this region of the protein is postulated to be cleaved by endocytic proteases during viral entry (32). It is possible that the tyrosine-to-histidine mutation at amino acid 354 enhances susceptibility of σ3 to proteolysis by directly altering the conformation of the σ3 cleavage site or by allowing σ3 to be cleaved by proteases that are not inhibited by E64. 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 proteolytic activity. In support of this hypothesis, it has been shown that interactions between σ3 and μ1 result in a conformational change in σ3 that increases its susceptibility to cleavage (32).

Reovirus entry is dependent on temporally associated acid-dependent and protease-dependent disassembly steps. Results of studies using reassortant viruses to identify genes that segregate with PI virus growth in cells treated with either ammonium chloride or E64 suggest that acidification and proteolysis mediate different events in reovirus disassembly. Growth of T1L × PI 2A1 and T1L × PI 3-1 reassortants in the presence of either ammonium chloride (40) or E64 (this report) segregates with the S4 gene. However, growth of T1L × L/C reassortants in the presence of ammonium chloride segregates with the S1 gene (40), whereas growth of T1L × L/C reassortants in the presence of E64 segregates with the S4 gene (this report). Thus, since growth in the presence of ammonium chloride segregates with either the S1 or S4 gene (Table 5), acid-dependent events appear to involve σ1, σ3, or both proteins. In contrast, studies described here identify the σ3 protein, and not the σ1 protein, as a determinant of susceptibility to viral growth inhibition by protease inhibitor E64. These findings suggest that susceptibility of the viral outer capsid to E64-sensitive proteolysis is an intrinsic property of the σ3 protein.

We compared kinetics of blockade produced by treatment of cells with ammonium chloride and E64 to determine whether acid-dependent and protease-dependent events are temporally related. We found that reovirus replication is susceptible to complete blockade by both ammonium chloride and E64 up to 30 min after viral adsorption; thereafter, susceptibility to both inhibitors decreases logarithmically for an additional 30 min. At times of addition greater than 60 min after adsorption, neither ammonium chloride nor E64 has a significant effect on reovirus growth. Together with results obtained in studies of PI viruses, these findings suggest that acid-dependent and protease-dependent events are discrete but temporally associated steps in reovirus disassembly and likely occur within the same cellular compartment. Therefore, our observations are consistent with a model in which endosomal acidification serves to induce conformational changes in viral outer-capsid proteins required for disassembly independent of effects on endocytic proteases.

In addition to σ3, outer-capsid protein μ1 is also cleaved during disassembly of reovirus virions to ISVPs. Cleavage of μ1 is thought to expose hydrophobic domains that facilitate interactions of ISVPs with vacuolar membranes (18, 27, 28). However, the μ1-encoding M2 gene was not shown to be a determinant of PI virus growth in cells treated with E64, using the reassortant viruses in the present study. Our results suggest that mutations in σ3 protein alone are sufficient to alter susceptibility of the viral outer capsid to proteolysis. It is possible that mutations are selected during persistent infection in both the σ3 and μ1 proteins; however, unlike mutations in σ3, mutations in μ1 alone might not be sufficient to alter susceptibility to proteolysis. Additionally, μ1 may play a role in reovirus disassembly subsequent to σ3 cleavage that is not affected

TABLE 5. 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 [19] and T1L × PI 2A1 and T1L × PI 3-1 reassortants [41]) in the presence of ammonium chloride (AC) (40) and in the presence of E64 (this report). Genes derived from PI viruses that segregate with mutant viral phenotypes are shown.

by E64 inhibition, and therefore μ1 would not be identified as a target for mutations conferring E64 resistance in these experiments.

Results of studies using reovirus mutants selected during persistent infection have identified viral structural proteins that mediate requirements for acidification and proteolysis during viral disassembly. Additionally, these studies have established the molecular basis for viral resistance to inhibitors of disassembly of virions to ISVPs (references 40 and 41 and this report). These studies suggest that disassembly of reovirus virions is initiated by acid-dependent processes affecting the σ1 and σ3 proteins and proteolysis of the σ3 protein. These events are followed in turn by proteolysis of μ1/μ1C to yield ISVPs, which are capable of membrane penetration. Ongoing studies of PI reoviruses will improve our understanding of the coordinated process of virus-cell interaction that culminates in stepwise disassembly of reovirus virions and activation of viral transcription.

ACKNOWLEDGMENTS

We thank Ken Tyler for assistance with the statistical analysis of the reassortant data. We express our appreciation to Erik Barton, Jim Chappell, Denise Wetzel, and Greg Wilson for careful review of the manuscript.

This work was supported by Public Health Service award T32 GM07347 from the National Institute of General Medical Studies for the Vanderbilt Medical-Scientist Training Program (G.S.B.), Public Health Service award AI32539 from the National Institute of Allergy and Infectious Diseases, and the Elizabeth B. Lamb Center for Pediatric Research.

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