A Carboxy-Terminal Fragment of Protein μ1/μ1C Is Present in Infectious Subvirion Particles of Mammalian Reoviruses and Is Proposed To Have a Role in Penetration

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Penetration of a cell membrane as an early event in infection of cells by mammalian reoviruses appears to require a particular type of viral particle, the infectious subvirion particle (ISVP), which is generated from an intact virion by proteolytic cleavage of the outer capsid proteins σ3 and μ1/μ1C. Characterizations of the structural components and properties of ISVPs are thus relevant to attempts to understand the mechanism of penetration by reoviruses. In this study, a novel, ~13-kDa carboxy-terminal fragment (given the name φ) was found to be generated from protein μ1/μ1C during in vitro treatments of virions with trypsin or chymotrypsin to yield ISVPs. With trypsin treatment, both the carboxy-terminal fragment φ and the amino-terminal fragment μ1δ/δ were shown to be generated and to remain attached to ISVPs in stoichiometric quantities. Sites of protease cleavage were identified in the deduced amino acid sequence of μ1 by determining the amino-terminal sequences of φ proteins: trypsin cleaves between arginine 584 and isoleucine 585, and chymotrypsin cleaves between tyrosine 581 and glycine 582. Findings in this study indicate that sequences in the φ portion of μ1/μ1C may participate in the unique functions attributed to ISVPs. Notably, the δ-φ cleavage junction was predicted to be flanked by a pair of long amphipathic α-helices. These amphipathic α-helices, together with the myristoyl group at the extreme amino terminus of μ1/μ1N, are proposed to interact directly with the lipid bilayer of a cell membrane during penetration by mammalian reoviruses.

The mechanisms by which different nonenveloped viruses penetrate cell membranes to enter the cytoplasm early in infection of cells are poorly understood. It is important to understand these mechanisms since the steps required to make viral particles competent for penetration and the steps in penetration itself are likely to serve as determinants of cell or tissue tropism and injury and thus to have relevance to studies of viral pathogenesis (33, 50). We have undertaken experiments that attempt to define this mechanism for one particular group of nonenveloped viruses, the mammalian reoviruses.

In this report, we provide further molecular characterization of the major outer capsid protein μ1 of reoviruses. The μ1 protein (76.3 kDa [18, 19, 53]; encoded by double-stranded RNA gene segment M2) occupies an important position in reovirus particles. In virions and infectious subvirion particles (ISVPs), μ1 occurs in ~600 copies (primarily in cleaved form as described below) and comprises a primary structural determinant of the $T = 13l$ icosahedral outer capsid (10, 23, 30, 31). Accumulating evidence (both genetic and biochemical) suggests that μ1 is an analog of the fusion proteins of enveloped viruses; it may interact directly with the lipid bilayer of a cell membrane during penetration and thus provide an essential function for initiating the cycle of replication in cells (28, 35, 37). In virions, viral protein σ3 (41.1 kDa; encoded by gene segment S4) is present in the outer capsid in association with (10, 27) and in equal numbers to (10, 18) μ1. The primary role of σ3 in assembled virions may be to stabilize the outer capsid until near the time when outer capsid proteins σ1 and μ1 effect attachment

to the surface of cells and penetration, respectively, to initiate infection (35). In fact, it appears that σ3 must be removed from reovirus particles by proteolysis (as has occurred in ISVPs) before penetration can occur (5, 28, 47). Other data suggest that μ1 must be lost from reovirus particles before a latent transcriptase activity in reovirus cores is activated to make the 10 full-length viral mRNAs (3, 9, 20, 44, 54). During replication in cells, this step may occur in conjunction with penetration or as a subsequent uncoating step within the cytoplasm (4, 6, 35, 37). Given these critical roles of the μ1 protein early in infection of cells, it is easy to see how μ1 might also be an important determinant of replication in host organisms and of pathogenesis (17, 51).

The μ1 protein is recognized to undergo two distinct proteolytic cleavages that may be important for regulating its functions. One cleavage occurs near its amino terminus and generates the small (4.2-kDa) amino-terminal fragment μ1N and the large (72.1-kDa) carboxy-terminal fragment μ1C (18, 37, 53). Both μ1 and μ1N are modified by N-myristoylation at their common amino terminus (37, 48). Since the cleavage of μ1 to μ1N and μ1C appears to occur during assembly of new viral particles in the cytoplasm of infected cells (56), most μ1 protein in mature virions appears to be present in the form of its two cleavage products (37); however, some uncleaved μ1 protein is also present in virions, suggesting that this cleavage is inefficient. We recently postulated that this cleavage is an autocatalytic one, on the basis of similarities between μ1 and the capsid polyproteins of picornaviruses (37). A role for σ3 in this cleavage was also postulated (41) and is the subject of current investigation (48). The μ1 protein undergoes an additional cleavage nearer its carboxy terminus during treatment of virions with exogenous endoproteases in vitro (3, 20, 44). A similar or identical

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cleavage also occurs within cultured cells (probably within late endosomes or lysosomes) at early times after infection (7, 45, 47) and in the lumen of the gut of newborn mice within minutes after peroral inoculation (1, 2). Both μ1 and μ1C are subject to this cleavage, which is recognized to generate the large (M_r 60,000 to 70,000) amino-terminal fragments μ1δ and δ, respectively (11, 18, 37). Recent data suggest that the latter cleavage is essential for penetration (28), consistent with its occurrence in many experimental settings. Based on the sizes of μ1δ and δ, the site of cleavage was estimated to be near residue 600 in the μ1 sequence (18).

This report describes new findings about the cleavage of μ1 during in vitro treatment of virions with exogenous endoproteases. Using treatment conditions that yield ISVPs, we identified that cleavage of μ1/μ1C by trypsin or chymotrypsin generates not only the large amino-terminal fragment μ1δ/δ but also a stable smaller (~13,000-M_r) carboxy-terminal fragment, which we have named φ. This fragment was not recognized in previous studies. The sites of cleavage by these proteases were localized in the μ1 sequence by amino-terminal sequencing of the respectively generated φ proteins: trypsin cleaves between residues 584 and 585, and chymotrypsin cleaves between residues 581 and 582. For treatment with trypsin, we were able to show that both μ1δ/δ and φ are generated and remain attached to ISVPs in stoichiometric quantities. Features of the μ1 sequence in a region surrounding the δ-φ cleavage junction are suggested to be important for the role of μ1 in penetration of a cell membrane as an early event in infection of cells by reoviruses.

MATERIALS AND METHODS

Cells and viruses. Stock cultures of murine L cells were grown in suspension in Joklik modified minimal essential medium (Irvine Scientific, Irvine, Calif.) supplemented to contain 2.5% fetal bovine serum (HyClone Laboratories, Logan, Utah), 2.5% neonatal bovine serum (Biocell Laboratories, Carson, Calif.), and 2 mM glutamine (Irvine Scientific). Reovirus strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) were the viruses used in this study. First- to third-passage lysate stocks of reoviruses were prepared by using monolayer cultures of L cells in the above medium plus 1% Fungi-Bact (Irvine Scientific). Plaque assays to isolate and titrate viruses were performed as described previously (13).

Preparation of purified virions. Purified virions were obtained as described previously (13) except that third-passage lysate stocks were generally used to initiate infections. Virion storage buffer consisted of 150 mM NaCl, 10 mM MgCl₂, and 10 mM Tris (pH 7.5). Particle concentrations of purified virion preparations were determined as described previously (46). To obtain purified virions containing [³⁵S]methionine-labeled or [³⁵S]cysteine-labeled proteins, we added Tran³⁵S-label (ICN Biochemicals, Costa Mesa, Calif.) or [³⁵S]cysteine (ICN Biochemicals) to the suspension at the initiation of infection (~12.5 μCi/ml). Tran³⁵S-label contains a relatively large proportion of [³⁵S]cysteine (≤15%) in addition to [³⁵S]methionine (≥70%). To obtain purified virions containing [³H]myristate-labeled proteins, we added [³H]myristic acid (Dupont, NEN Research Products, Boston, Mass.) to the cell suspension at the initiation of infection (~12.5 μCi/ml).

ISVPs and cores. ISVPs and cores were obtained by treating purified virions at designated concentrations in vitro with either 100 μg of Nα-p-tosyl-L-sulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated bovine trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml or 200 μg of Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine α-chymotrypsin (Sigma Chemical Co.) per ml for designated times. All treatments were done in virion storage buffer at 32°C and were ended by additions of 5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and 300 μg of soybean trypsin inhibitor (Sigma Chemical Co.) per ml and removal of the treatment mixture to 0 to 4°C. Additional amounts of the trypsin inhibitor TLCK were added to some treatments with chymotrypsin as described in Results. In many cases, treatment mixtures were prepared directly for electrophoresis as described below. In other cases, ISVPs and cores were isolated from the treatment mixtures by equilibrium sedimentation in preformed CsCl density gradients as described previously (13) except that centrifugation was performed with either a Beckman 28.1 rotor at 25,000 rpm for 4 to 16 h or a Beckman 50.1 rotor at 40,000 rpm for 2 to 6 h at 5°C. Before electrophoresis, viral particles were sometimes concentrated by pelleting in a Beckman 50.1 rotor at 40,000 rpm for 1 h at 5°C and resuspended in virion storage buffer.

SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with gradient gels (15) was performed as described previously (18). Unless otherwise noted, samples in virion storage buffer (treatment mixtures or isolated particles) were prepared for electrophoresis by diluting 1:1 with 2× sample buffer (250 mM Tris [pH 8.0], 4% 2-mercaptoethanol, 2% SDS, 0.02% bromophenol blue) and heating in a boiling water bath for 1 to 2 min. Gels containing radiolabeled proteins were generally treated with Enlightning (NEN Research Products), dried onto filter paper under vacuum, and exposed to RX film (Fuji Photo Film, Tokyo, Japan) at room temperature or −70°C. Coomassie brilliant blue-stained gels were dried between sheets of cellophane (Bio-Rad Laboratories, Richmond, Calif.). Estimates of the relative molecular weights (M_rs) of proteins were determined by comparisons with marker proteins (Bethesda Research Laboratories, Gaithersburg, Md.; Sigma Chemical Co.) by using linear regression analysis.

Amino-terminal sequencing. For SDS-PAGE of samples for amino-terminal sequencing, the separating gel was subjected to pre-electrophoresis overnight before pouring the stacking gel; in addition, 0.1 mM sodium thioglycolate (Sigma Chemical Co.) was added to cathode buffer overlying the stacking gel and a short pre-electrophoresis was performed (enough to permit the ion front to clear the sample wells) before virus-containing samples were loaded. Otherwise, SDS-PAGE was performed as described above. After electrophoresis, proteins were subjected to electroblotting onto polyvinylidene difluoride (PVDF) paper (Immobilon P from Millipore Corp., Bedford, Mass., or ProBlott from Applied Biosystems, Foster City, Calif.) as described previously (29). Briefly, the gel was first soaked for 5 min in transfer buffer (10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid; pH 11.0] in 10% methanol), placed in contact with PVDF paper, and assembled into an electroblotting apparatus with plate electrodes (Idea Scientific, St. Paul, Minn.). Transfer was done in transfer buffer at 24 V for 30 to 45 min. The electroblot was then stained with Ponceau S (0.2% in 1% acetic acid) and destained briefly with deionized water. Protein bands desired for amino-terminal sequencing were excised and stored at −20°C until analysis. Amino-terminal sequencing was performed at the Harvard Microchemistry Facility according to standard protocols. Briefly, Edman degradation of samples was done in a 470A

gas-phase protein sequencer (Applied Biosystems), and re-leased, phenylthiohydantoin (PTH)-derivatized amino acids were analyzed on-line with a 120A HPLC analyzer (Applied Biosystems) and a C-R4A integrator (Shimadzu, Columbia, Md.). First-cycle yields in these determinations ranged be-tween 99 and 186 pmol.

Quantitative analysis of viral proteins. Quantitation exper-iments were performed similarly to those described previ-ously (18). Individual [^{35}S]cysteine-labeled protein bands were excised from stained gels that had been dried between cellophane and were solubilized by incubation overnight at 65°C in sealed tubes containing 0.5 ml of 30% H_2O_2 (15). After cooling, 200 μl of each sample was mixed with 4 ml of Scintiverse II (Fisher Scientific Co., Fair Lawn, N.J.) and allowed to sit at room temperature for several hours to permit dissipation of chemiluminescence. Radioactivity was then measured with a Beckman LS-233 scintillation counter. Blank gel slices (not containing protein) were handled in an identical manner and used to provide values for background radioactivity. The amount of radioactivity associated with the σ2 protein in each lane was used to standardize values for the μ1, μ1C, μ1δ, δ, and/or φ proteins in that lane. As an internal control for these experiments, the ratio of [^{35}S]cys-teine-associated radioactivity in proteins σ3 and μ1 plus μ1C from samples of virions ($n = 14$) was determined to be 1.50 (± 0.03); correction for the number of cysteine residues in the two proteins implies a molar ratio of 1.00 for σ3 and μ1 plus μ1C, as indicated in a previous study (18).

Computer-based sequence analyses. All computer-based sequence analyses were performed through the Whitaker College Computing Facility at the Massachusetts Institute of Technology. The programs PEPTIDESORT, ISOELEC-TRIC, and PEPTIDESTRUCTURE from the sequence anal-ysis software package of the University of Wisconsin Genet-ics Computer Group were utilized. For studies with PEPTIDESTRUCTURE, default parameters were accepted for assigning unique predictions of secondary structure to individual residues in μ1.

RESULTS

Stable 13,000-M_r protein is generated during conversion of virions to ISVPs by trypsin. Purified [^{35}S]methionine-labeled virions of reovirus strain T1L were treated for different times with trypsin under conditions that yield ISVPs (rather than cores) as end products. When the samples were examined by SDS-PAGE directly (i.e., without first separating viral par-ticles from solubilized cleavage products), evidence for a series of proteolytic events was seen (Fig. 1A). The major outer capsid protein σ3 was rapidly degraded. Loss of the σ3 band was accompanied by the appearance of low-molecular-weight fragments, which were seen transiently but had mostly disappeared by the 5-min timepoint (Fig. 1A, lanes 2 to 4). Beginning between 1 and 2 min of treatment (Fig. 1A, lanes 2 and 3), cleavage of the μ1 and μ1C proteins was also observed, as represented by decreasing intensity of the μ1 and μ1C bands and increasing intensity of two new bands of lower molecular weight that were suggestive of the μ1δ and δ proteins that have been identified after chymotrypsin treatment of virions (Fig. 2); (11, 18, 37). The T1L σ1 protein was not cleaved by trypsin in these experiments, consistent with prior observations (2, 55).

Most pertinent to the current study, a ^{35}S-labeled protein of M_r ~13,000 was first noted in the 5-min sample in this experiment and remained present throughout the entire 60-min treatment with trypsin (Fig. 1A, lanes 3 to 8). In
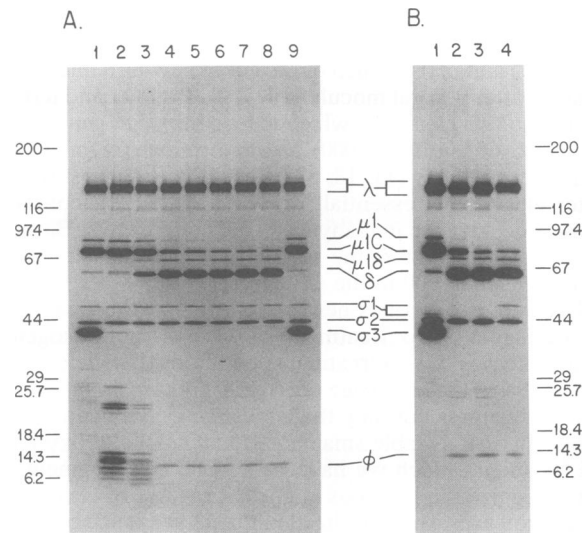

FIG. 1. Cleavage of viral proteins σ3 and μ1/μ1C during treat-ments of virions with trypsin to generate ISVPs. (A) Purified, [^{35}S]methionine-labeled virions of reovirus T1L at a concentration of 2 × 10^{12} particles per ml were treated with trypsin for different times under conditions that yield ISVPs as end products, and samples were prepared for electrophoresis directly. Equal volumes of samples (~20 μl) were loaded into wells atop an SDS-polyacryl-amide gradient gel. After electrophoresis, the gel was prepared for fluorography and exposed to film for 4 days at room temperature. Times (min) of treatment with trypsin: 0 (lane 1), 1 (lane 2), 2 (lane 3), 5 (lane 4), 10 (lane 5), 20 (lane 6), 40 (lane 7), and 60 (lane 8). A sample of untreated virions appears in lane 9. (B) Purified, [^{35}S]me-thionine-labeled virions of reovirus T3D (lanes 1 to 3) or T1L (lane 4) were treated with trypsin and handled as described above. The gel was exposed to film for 7 days at −70°C. Times (minutes) of treatment with trypsin: 0 (lane 1), 5 (lane 2), and 60 (lanes 3 and 4). Viral proteins are indicated in the space between the gels. Positions of marker proteins in each gel are indicated by their M_rs (×10^3) at left and right. The φ band represents the novel protein fragment characterized in this report.

subsequent experiments, this band was found to be un-changed in mobility and intensity after as much as 3 h of trypsin treatment (data not shown). Such a protein has not been recognized in prior studies involving treatment of reovirus particles with proteases. To address whether the protein is specific to reovirus T1L, we treated purified [^{35}S]methionine-labeled virions of reovirus T3D with trypsin under conditions that yield ISVPs of that strain as end products (Fig. 1B). Once again, a 13,000-M_r protein was observed by SDS-PAGE which was stable over a 60-min course of trypsin treatment and comigrated with the 13,000-M_r protein of reovirus T1L (Fig. 1B, lanes 3 and 4). A 13,000-M_r protein was also observed after a 20-min treat-ment of reovirus strain T2J with trypsin (data not shown). For ease of discussion, we will subsequently refer to this novel protein, which is common to prototypical strains representing all three reovirus serotypes, as the φ protein.

There are two likely possibilities for the identity of φ: a fragment of σ3 or a sister fragment to μ1δ/δ resulting from the cleavage of μ1/μ1C. With regard to the second possibil-ity, since μ1δ/δ is known to be an amino-terminal fragment of μ1/μ1C when resulting from cleavage by chymotrypsin (18, 37), the φ protein would likely be a carboxy-terminal fragment (Fig. 2). To determine whether φ is the correct approximate size to be such a fragment of μ1/μ1C, we
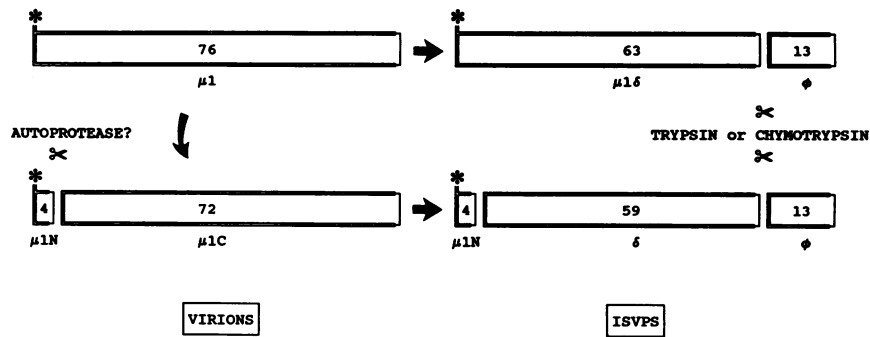
FIG. 2. Diagrams of μ1 protein highlighting its cleavages and fragments. Rectangles represent the μ1 protein or its fragments and are drawn to approximate scale for the molecular mass (in kilodaltons) of each, determined as described in the text and indicated within each rectangle. A cleavage near the amino terminus of μ1, generating the μ1N and μ1C fragments, has been proposed to result from an autoproteolytic activity inherent to structural features of the μ1 protein or other viral components (37). The myristoyl group at the extreme amino terminus shared by μ1 and fragments μ1N and μ1δ is indicated with an asterisk. As shown in this study, cleavage of μ1 or μ1C by exogenous proteases, such as trypsin or chymotrypsin, generates the amino-terminal fragment μ1δ or δ, respectively, and the carboxy-terminal fragment φ. The carboxy termini of μ1 and its proteolytic fragments have yet to be defined precisely, as indicated by single lines in these diagrams. The μ1 protein appears to take primarily the form of μ1N and μ1C (plus a small amount of intact μ1) in virions and μ1N, δ, and φ (plus a small amount of μ1δ) in ISVPs generated by protease treatments for sufficient times (Fig. 1).

estimated the sizes of the T1L μ1, μ1C, μ1δ, and δ proteins from several 5 to 20% gradient gels (data not shown). The differences in M_r between the μ1 and μ1δ proteins (13,900) and between the μ1C and δ proteins (13,700) were noted to approximate the size of the φ protein (Fig. 1); however, since φ migrates in the nonlinear region (15) near the bottom of 5 to 20% gradient gels, an accurate M_r could not be assigned to it in this manner.

Amino-terminal sequencing of 13,000-M_r φ protein identifies it as a carboxy-terminal fragment of μ1/μ1C. To identify whether φ is derived from μ1/μ1C or σ3, we opted to determine its amino-terminal sequence. We recognized that this approach would be simplified if φ is not released from ISVPs after cleavage, but instead remains particle associated so that it can be isolated along with ISVPs. To test for this possibility, we treated purified nonradiolabeled virions of reovirus T1L with trypsin for 60 min, and the resulting ISVPs were isolated by centrifugation in a CsCl density gradient. After dialysis in virion storage buffer, ISVPs from an aliquot of this material were concentrated by pelleting in the ultracentrifuge and examined by SDS-PAGE (Fig. 3A). A 13,000-M_r protein was clearly visible in this sample (Fig. 3A, lane 1). Although this experiment demonstrates that many copies of φ remain attached to ISVPs, it does not prove that φ is generated or remains attached in stoichiometric quantities (see below).

Aided by the fact that significant quantities of the φ protein cosediment with ISVPs, we proceeded with its amino-terminal sequencing. Gradient-isolated T1L ISVPs obtained as described above were concentrated by pelleting in the ultracentrifuge and subjected to SDS-PAGE. Proteins from the gel were transferred onto PVDF paper by electroblotting and visualized by staining with either Coomassie brilliant blue (Fig. 3B) or Ponceau S. Protein bands stained with Ponceau S were excised and placed into the sample cup of the gas-phase sequenator for sequence analysis via Edman degradation. In this manner, the amino-terminal sequence of the φ protein was determined to be isoleucine-phenylalanine-asparagine-proline-lysine (IFNPK) corresponding to amino acids 585 to 589 in the deduced sequence of the T1L μ1 protein (19, 53) (Table 1). Isoleucine 585 is immediately preceded in the T1L μ1 sequence by arginine 584, consistent with φ resulting from a cleavage by trypsin,

which cleaves after arginine and lysine residues. An endoproteolytic cleavage by trypsin at this site, assuming no additional cleavages near the carboxy terminus of μ1/μ1C, would yield a φ protein with a calculated mass of 13,300 Da.
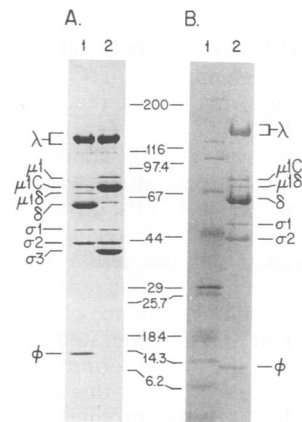

FIG. 3. (A) Proteins in gradient-isolated, trypsin-generated ISVPs and (B) electroblotting of viral proteins in preparation for amino-terminal sequencing of φ and δ. (A) Purified, nonradiolabeled virions of reovirus T1L at a concentration of 10^{13} particles per ml were treated with trypsin for 60 min. ISVPs were isolated from the treatment mixture by centrifugation in a CsCl density gradient, dialyzed extensively in virion storage buffer, concentrated by pelleting in the ultracentrifuge, and prepared for electrophoresis (lane 1). An aliquot of untreated virions was concentrated by pelleting and prepared for electrophoresis in the same manner (lane 2). For each sample, ~1.5 × 10^{12} particles were loaded atop the SDS-polyacrylamide gradient gel. After electrophoresis, proteins were stained with Coomassie brilliant blue. (B) Sample containing ~1.5 × 10^{12} T1L ISVPs was prepared as described above and loaded atop an SDS-polyacrylamide gradient gel that had been subjected to pre-electrophoresis. After electrophoresis, proteins were transferred to PVDF paper by electroblotting and stained with Coomassie brilliant blue (electroblots stained with Ponceau S in other experiments were comparable). Marker proteins (lane 1) and T1L ISVPs (lane 2) are shown. Positions of marker proteins in each gel are indicated by their M_rs (×10^3) in the space between the gels. Viral proteins are indicated at left and right.

TABLE 1. Amino-terminal sequence determinations for proteins from virions and ISVPs of reovirus T1L

Protein	Protease (min)[a]	Amino-terminal sequence[b]
μ1	None	Myr[c]
μ1C	None	43-PGGVP[d]
μ1δ	TRY (30)	Myr
δ	TRY (60)	43-PGGVP
φ	TRY (60)	585-IFNPK
μ1δ	CHT (30, 37°C)	Myr[c]
δ	CHT (40, 37°C)	43-PGGVP[d]
φ	CHT (5)	582-GVRIF, 585-IFNPK (14, 1)
φ[e]	CHT (60)	582-GVRIF, 585-IFNPK (1, 4)
φ[f]	CHT (60)	585-IFNPK

[a] TRY, trypsin; CHT, chymotrypsin. Time for which virions were treated with protease appears in parentheses (treatment at 32°C unless stated otherwise).

[b] Myr, protein modified with an N-myristoyl group. Amino-terminal sequences are designated by single-letter amino acid code preceded by the position number of the first residue in the deduced T1L μ1 sequence. If two amino termini were identified in the sample, both are given, followed by their approximate relative quantities in parentheses.

[c] From reference 37.

[d] From reference 18.

[e] Upper and middle φ bands in 60-min chymotrypsin samples (as seen in Fig. 6B, lane 4, and C, lane 2) were not easily separated and were submitted together for amino-terminal sequencing.

[f] Lower φ band in 60-min chymotrypsin sample (as seen in Fig. 6B, lane 4, and C, lane 2) was submitted for amino-terminal sequencing separately from the upper and middle bands.
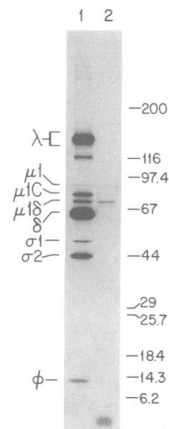

FIG. 4. Myristoylated proteins in ISVPs generated from virions by treatment with trypsin. Purified, [35S]methionine-labeled (lane 1) or [3H]myristate-labeled (lane 2) virions of reovirus T1L at a concentration of 10^13 particles per ml were treated with trypsin for 30 min, and samples were prepared for electrophoresis directly. The 35S-labeled sample was diluted 1:9 with sample buffer, and equal volumes of samples (~20 μl) were then loaded atop an SDS-polyacrylamide gradient gel. After electrophoresis, the gel was prepared for fluorography and exposed to film for 14 days at −70°C. Positions of marker proteins are indicated by their M_rs (×10^3) at right. Viral proteins are indicated at left.

Thus, the 13,000-M_r φ protein is likely to represent the carboxy-terminal fragment of μ1/μ1C generated by the same proteolytic cleavage that yields the larger amino-terminal fragment μ1δ/δ (Fig. 2).

Trypsin does not cleave near the amino terminus of μ1/μ1C. A stated aim of the current study was to characterize the structural components of ISVPs. In this regard, we recognized that trypsin might also cleave near the amino terminus of μ1/μ1C in generating the μ1δ/δ fragment of trypsin-generated ISVPs. To address this possibility, we first determined the amino-terminal sequence of the δ protein from the same PVDF blot of ISVPs generated by a 60-min trypsin treatment as yielded the amino-terminal sequence of φ. The amino-terminal sequence of δ in this case was identified as proline-glycine-glycine-valine-proline (PGGVP), corresponding to amino acids 43 to 47 in the deduced T1L μ1 sequence and also corresponding to the amino-terminal sequence determined previously for the T1L μ1C protein (18) (Table 1). Given that μ1C and trypsin-generated δ share the same amino terminus, trypsin appears not to cleave near the amino terminus of μ1C.

The μ1 protein is modified by N-myristoylation at its extreme amino terminus (37, 48), which should block it from amino-terminal sequence analysis; nonetheless, the myristoyl group can serve as a marker for whether trypsin cleaves near the amino terminus of μ1 in generating μ1δ. [3H]myristate-labeled T1L virions were treated with trypsin for 30 min and examined by SDS-PAGE. Most of the radioactivity in such [3H]myristate-labeled samples migrates with the ion front in association with the μ1N protein (37) (Fig. 4, lane 2); however, a distinct 3H-labeled protein was also noted in this sample, comigrating with the μ1δ protein in a sample of identically treated [35S]methionine-labeled T1L virions (Fig. 4, lane 1). Thus, μ1 and trypsin-generated μ1δ appear to share the same amino-terminal myristoyl group,

indicating that trypsin has not cleaved near the amino terminus of μ1. Previous experiments indicated that chymotrypsin also does not cleave near the amino terminus of either μ1C (18) or μ1 (37) (Table 1).

Stoichiometric quantities of φ and μ1δ/δ remain attached to ISVPs generated with trypsin. We wished to determine whether all μ1/μ1C molecules that undergo cleavage when ISVPs are made with trypsin yield stable μ1δ/δ and φ fragments and also whether these fragments remain attached to ISVPs in stoichiometric quantities. For this purpose, purified virions of reovirus T1L were obtained after metabolic labeling with [35S]cysteine (Fig. 5, lane 1). Labeling with [35S]cysteine was chosen for several reasons, including the relatively large portion of μ1/μ1C-associated radiolabel that was expected to segregate with φ after cleavage: φ should contain 1 of only 4 cysteine residues deduced to be present in both μ1 and μ1C (versus only 1 of 16 and 15 methionines in T1L μ1 and μ1C, respectively) (19, 37, 53).

Amounts of 35S associated with the viral structural proteins in either virions or ISVPs were determined after separating the proteins by SDS-PAGE, excising individual protein bands from stained and dried gels, solubilizing the gel fragments with hydrogen peroxide, and measuring radioactivity by liquid scintillation counting (Table 2). To correct for variations in the loading of samples between lanes and in different gels, radioactivity values for the proteins of interest in each lane (μ1-derived proteins) were expressed relative to the value obtained for the σ2 protein in that lane. Similar results were obtained when correction was performed relative to the λ protein band in each lane (data not shown).

In all experiments, an amount of radioactivity approximating the total from the μ1 and μ1C proteins of virions was recovered in the μ1δ, δ, φ, and uncleaved μ1C proteins of samples containing ISVPs, indicating that little or none of the μ1/μ1C-associated [35S]cysteine was lost because of nonspecific degradation of these proteins (Table 2). In a pair of initial experiments, [35S]cysteine-labeled T1L virions
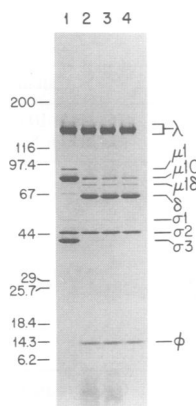
FIG. 5. Proteins in [^{35}S]cysteine-labeled virions and trypsin-generated ISVP samples such as used in stoichiometric determinations of φ and δ. Samples and gel were handled in a manner identical to that summarized in Table 2 except that the gel was destained for 1 h rather than 15 min and dried onto filter paper for autoradiography rather than dried in cellophane. Samples containing approximately 2 × 10^{11} particles were loaded atop an SDS-polyacrylamide gradient gel and subjected to electrophoresis. A sample containing intact virions (lane 1) was prepared for electrophoresis without additional treatment. Trypsin-treated samples were obtained by treating purified, radiolabeled virions at a concentration of 10^{13} particles per ml with trypsin for either 20 (lane 2) or 60 (lane 3) min before preparing them for electrophoresis. A sample containing gradient-isolated ISVPs that had been obtained for the quantitation experiments after treatment of purified, radiolabeled virions at a concentration of 10^{13} particles per ml with trypsin for 60 min (lane 4) was prepared for electrophoresis without additional treatment. The dried gel was exposed to film for 7 days at room temperature. Positions of marker proteins are indicated by their M_rs (×10^3) at left. Viral proteins are indicated at right. Low-molecular-weight degradation products of σ3 are present in the trypsin-treated samples (lanes 2 and 3).

were treated with trypsin for either 20 or 60 min, and these ISVP-containing samples were subjected to SDS-PAGE (Fig. 5, lanes 2 and 3). In each of these cases, of the radioactivity lost from the μ1 and μ1C proteins in ISVP-containing samples versus virions, approximately 25% was recovered in association with the φ protein and approximately 75% was recovered in association with the μ1δ and δ bands (Table 2), consistent with the expected number of cysteine residues in these fragments. For a final pair of experiments, [^{35}S]cysteine-labeled T1L virions were treated with trypsin for 60 min, and the resulting ISVPs were purified by centrifugation in a CsCl density gradient, dialyzed in storage buffer, and subjected to SDS-PAGE (Fig. 5, lane 4). Again in this case, approximately 25% of the radioactivity lost from the μ1 and μ1C proteins in ISVPs versus virions was recovered in association with the φ protein and approximately 75% was recovered in association with the μ1δ and δ proteins (Table 2). These experiments strongly suggest that most or all of the μ1/μ1C molecules that are cleaved during the generation of ISVPs with trypsin yield stable μ1δ/δ and φ fragments and that most or all of these fragments remain attached to ISVPs. Significant amounts of φ protein were also found to remain attached to ISVPs obtained after protease treatments of virions of the other prototype strains T2J and T3D, but we have not subjected those specimens to the rigorous quantitative analysis presented here for strain T1L.

φ protein generated by chymotrypsin treatment is subject to additional cleavages near its termini. Since chymotrypsin,

rather than trypsin, was used to make ISVPs in most prior studies (e.g., see reference 47), we wished to analyze the φ protein generated during chymotrypsin treatment. In an initial experiment, [^{35}S]methionine-labeled T1L virions were treated with chymotrypsin, and multiple samples from a 60-min treatment were analyzed by SDS-PAGE (Fig. 6A). A 13,000-M_r protein was first seen at the 5-min timepoint (Fig. 6, lane 4); however, in contrast to trypsin treatment, the protein was noted to change mobility at subsequent timepoints. By 60 min of treatment, two additional bands were present, both having faster mobilities than the original 13,000-M_r protein (Fig. 6A, lane 8). These findings indicated that additional cleavages can occur near one or both ends of the φ protein that is initially generated by chymotrypsin.

To determine at which site chymotrypsin first cleaves the μ1 protein, we obtained amino-terminal sequences of φ proteins from ISVPs isolated after 5 min of treatment with chymotrypsin. This approach was again aided by the fact that chymotrypsin-generated φ proteins remain attached to ISVPs after isolation in CsCl density gradients, dialysis, and pelleting (data not shown). The amino terminus of φ protein generated by 5 min of chymotrypsin treatment was determined to be glycine-valine-arginine-isoleucine-phenylalanine (GVRIF) (Table 1), corresponding to amino acids 582 to 586 in the deduced sequence of T1L μ1 protein (18, 53). Glycine 582 is immediately preceded in the T1L μ1 sequence by tyrosine 581, consistent with φ resulting from an initial cleavage by chymotrypsin, which cleaves preferably after aromatic residues like tyrosine. Thus, trypsin and chymotrypsin cleave at sites separated by only three residues in the μ1 sequence (Fig. 7).

A notable finding was that a second amino terminus, IFNPK, was also found in the sample of φ protein obtained after 5 min of chymotrypsin treatment, albeit in only an ~1:14 ratio with the first amino terminus, GVRIF (Table 1). This second amino terminus is the same as that resulting from trypsin treatment, and its occurrence suggested that a trypsinlike protease (possibly trypsin itself) was contaminating the stock of chymotrypsin used in these experiments and contributed to the changing mobility of the φ protein observed at later times of chymotrypsin treatment by effecting an additional cleavage between residues 584 and 585 near the amino terminus of the φ protein first generated by chymotrypsin cleavage between residues 581 and 582. This idea was reinforced by amino-terminal sequence analyses of the φ proteins obtained after 60 min of treatment with chymotrypsin: the closely migrating upper and middle bands at 60 min were analyzed together and were determined to include the two amino termini GVRIF and IFNPK in an ~1:4 ratio, and the lower band was analyzed separately and was determined to include the single amino terminus IFNPK (Table 1). Using Nα-p-tosyl-L-arginine methyl ester (TAME) as a colorimetric substrate, we were able to show that stocks of chymotrypsin from two different commercial lots contained similar amounts of trypsinlike activity: 0.4 and 0.2 TAME U/mg of protein (versus 950 TAME U/mg of protein in the trypsin stock used in these studies). Trypsin contamination had occurred despite the fact that the chymotrypsin was treated during its commercial preparation with the trypsin inhibitor TLCK.

We reasoned that contaminating trypsinlike protease might be inhibited by adding more TLCK to the chymotrypsin treatment mixtures. A dose of 125 μM TLCK was found to have little or no effect on the pattern of cleavage of viral proteins other than φ. After 5 min of chymotrypsin treatment of [^{35}S]methionine-labeled T1L virions, the mobil-

TABLE 2. Quantitative experiments with [35S]cysteine-labeled virions and ISVPs of reovirus T1L to determine yields of μ1δ/δ and φ after trypsin treatment

Expts^a	Sample (min)^b	Experimentally determined value (±SD)^c				Calculated value^d			
		n	μ1 + μ1C (A)	μ1δ + δ (B)	φ (C)	A*	(B/A*) × 100 (% μ1δ + δ)	(C/A*) × 100 (% φ)	R
1 and 2	Virions	6	1.94 (± 0.05)	NA^e	NA	NA	NA	NA	NA
	ISVPs (20)	3	0.21 (± 0.02)	1.32 (± 0.04)	0.40 (± 0.02)	1.73	76	23	99
	ISVPs (60)	6	0.17 (± 0.01)	1.35 (± 0.05)	0.43 (± 0.01)	1.76	76	24	100
3 and 4	Virions	8	1.94 (± 0.04)	NA	NA	NA	NA	NA	NA
	ISVPs (60)	8	0.15 (± 0.01)	1.42 (± 0.02)	0.43 (± 0.02)	1.79	79	24	103
	Expected^f		NA	NA	NA	NA	75	25	100

^a Treatment mixtures were used as sources of ISVPs in experiments 1 and 2; ISVPs isolated by centrifugation in CsCl gradients were used in experiments 3 and 4.

^b Time of treatment with trypsin used to generate ISVPs is indicated.

^c Experimental values were determined by scintillation counting of solubilized gel slices. Values for μ1-derived proteins (A, B, and C) represent averages for the number (n) of each type of sample as determined after standardization relative to the amount of σ2 protein in each sample.

^d A* (applicable to ISVP-containing samples) = A (virions) − A (ISVPs), or the quantity of μ1 and μ1C in virions that was cleaved by trypsin and should be accounted for in μ1δ, δ, and φ in each type of ISVP-containing sample. (B/A*) × 100, percentage of counts from cleaved μ1 and μ1C that was recovered in μ1δ and δ. (C/A*) × 100, percentage of counts from cleaved μ1 and μ1C that was recovered in φ. R, recovery of radioactivity from μ1C, μ1δ, δ, and φ in ISVP-containing samples as a percentage of the radioactivity from μ1 and μ1C in virions.

^e NA, not applicable.

^f Since μ1 and μ1C contain four cysteines, μ1δ and δ contain three cysteines, and φ contains one cysteine, the value (B/A*) × 100 should equal (3/4) × 100 or 75, and the value (C/A*) × 100 should equal (1/4) × 100, or 25, if μ1δ/δ and φ are generated (experiments 1 and 2) or both are generated and remain attached to ISVPs (experiments 3 and 4) in stoichiometric quantities.

ity of φ protein was the same whether or not 125 μM TLCK was present during treatment (Fig. 6B, lanes 1 and 2), but after 60 min of treatment, different patterns were observed (Fig. 6B, lanes 3 and 4). In the presence of TLCK, two φ bands were generated in similar relative amounts as the middle and lower bands generated in the absence of TLCK; however, the upper of these two bands now comigrated with the φ protein seen after 5 min of treatment. The lower of the two bands generated in the presence of TLCK also had a changed (slower) mobility. Identical observations were made when [35S]cysteine-labeled T1L virions were treated with chymotrypsin with or without TLCK for 5 or 60 min (Fig. 6C). Also, very similar results were obtained when [35S]methionine-labeled virions of reovirus T3D were treated with chymotrypsin to generate ISVPs in either the absence or presence of TLCK (Fig. 6D). These findings indicate that although additional cleavage near the amino terminus of the chymotrypsin-generated φ protein by a contaminating trypsinlike protease was inhibited by TLCK, another additional cleavage near the carboxy terminus of φ was not inhibited and is therefore likely to be effected by chymotrypsin or a distinct contaminating protease. Studies in progress are designed to define more precisely the sites of protease cleavage near the carboxy terminus of φ (36); however, the continued radiolabeling of all fragments with [35S]cysteine (Fig. 6C) indicates that such cleavages do not occur amino terminal to the single cysteine residue in φ, cysteine 679, which occurs 29 residues from the carboxy terminus of μ1 (19, 53).

Trypsin- and chymotrypsin-generated φ proteins are calculated to have highly basic isoelectric points. Having identified the amino termini of φ proteins generated with trypsin and chymotrypsin, we undertook computer-based studies to identify unique features of sequences composing the φ region of μ1. One such study concerned the isoelectric points (pIs) of φ and other fragments of μ1, as calculated by using the program ISOELECTRIC and the deduced amino acid sequences of μ1 proteins from reovirus strain T1L (Table 3) or T3D (data not shown, but very similar). The T1L

μ1 protein and its fragments μ1N and μ1C are acidic, having pIs of 4.8, 3.6, and 4.9, respectively, according to calculations from their sequences; however, acidic and basic amino acids are not evenly distributed within the large μ1C fragment. The φ portion of μ1C is highly basic, having a calculated pI of ~11, and consequently the δ portion of μ1C is more acidic than the parent molecule, having a calculated pI of 4.4. For the T1L φ and δ fragments generated by trypsin cleavage, the large difference in pI reflects a ±32 difference in net charge of the two fragments (Table 3), despite the fact that charged residues in total make up similar portions of their sequences (22 and 20% for φ and δ, respectively). Because of the distribution of charged residues in μ1C, additional cleavages that might occur near the carboxy terminus of φ or δ would not have major effects on the pI of either (data not shown). The remarkable difference in charge between φ and δ is discussed more below.

Amphipathic α-helices are predicted to flank the δ-φ junction. The deduced amino acid sequences of μ1 proteins from reoviruses T1L and T3D were also subjected to computer-based techniques for predicting secondary structures in an attempt to identify interesting features near the δ-φ junction region of μ1. Cleavage by trypsin and chymotrypsin occurs in a region of μ1 sequence that is predicted to form β-strands and β-turns; however, close to the carboxy-terminal side of the cleavage site lies a region with strong α-helical predictions (Fig. 7). When positioned on a helical wheel (42), residues 591 to 604 in particular appear to form an α-helix that is distinctly amphipathic, including an apolar face that covers at least half of the α-helical surface (Fig. 7). A second amphipathic α-helix, even longer than the first and having an apolar face that covers about one-half of the α-helical surface, is predicted between residues 534 and 551 on the amino-terminal side of the δ-φ cleavage junction (Fig. 7). This amphipathic α-helix near the carboxy terminus of δ also has a striking distribution of charged residues on its polar face: charged residues are arranged so that basic residues occur on the sides of the α-helix adjacent to the apolar face and acidic residues occur on the side of the α-helix most
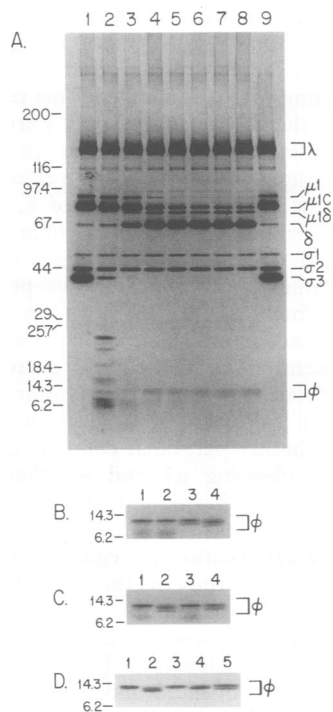
FIG. 6. Cleavage of viral proteins σ3 and μ1/μ1C during treatments of virions with chymotrypsin to generate ISVPs, with or without the presence of the trypsin inhibitor TLCK. (A) Purified, [³⁵S]methionine-labeled T1L virions at a concentration of 2 × 10¹² particles per ml were treated with chymotrypsin for different times in the absence of TLCK. Times (minutes) of treatment with chymotrypsin were: 0 (lane 1), 1 (lane 2), 2 (lane 3), 5 (lane 4), 10 (lane 5), 20 (lane 6), 40 (lane 7), and 60 (lane 8). A sample of untreated virions appears in lane 9. (B) Purified, [³⁵S]methionine-labeled T1L virions at a concentration of 10¹³ particles per ml were treated with chymotrypsin for 5 (lanes 1 and 2) or 60 (lanes 3 and 4) min in the absence (lanes 1 and 4) or presence (lanes 2 and 3) of 125 μM TLCK. (C) Purified, [³⁵S]cysteine-labeled T1L virions at a concentration of 10¹³ particles per ml were treated with chymotrypsin for 5 (lanes 1 and 3) or 60 (lanes 2 and 4) min in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 125 μM TLCK. (D) [³⁵S]methionine-labeled T3D (lanes 1 to 4) or T1L (lane 5) virions at a concentration of 2 × 10¹² particles per ml were treated with chymotrypsin for 5 (lanes 1 and 3) or 60 (lanes 2, 4, and 5) min in the absence (lanes 1 and 2) or presence (lanes 3, 4, and 5) of 125 μM TLCK. For all four panels, samples were prepared for electrophoresis directly, and equal volumes of samples (~20 μl) were loaded atop SDS-polyacrylamide gradient gels. After electrophoresis, the gels were prepared for fluorography and exposed to film for 14 (A), 25 (B), 15 (C), or 5 (D) days at room temperature. Only the ϕ regions of the gels are shown in panels B and C. Positions of marker proteins are indicated by their M_rs (×10³) at left in each panel.

distal to the apolar face. The relevance of this pattern and the possible significance of amphipathic α-helices flanking the δ-ϕ junction for the proposed role of μ1 in penetration of the cell membrane during early events in infection are discussed below.

DISCUSSION

Virions and two types of subvirion particles, ISVPs and cores, are thought to perform specialized functions during replication of mammalian reoviruses in cells and host organisms (reviewed in reference 35). ISVPs in particular have
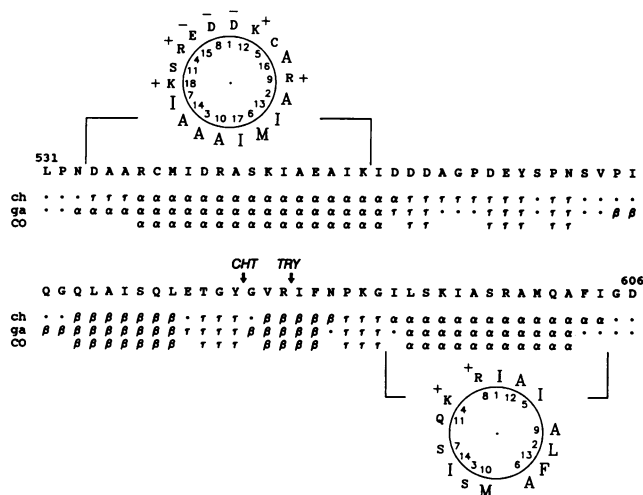

FIG. 7. Features of T1L μ1 protein in a region of sequence near the site of cleavage by exogenous proteases (amino acids 531 to 606). The deduced amino acid sequence (19, 53) is written in single-letter code. Positions of cleavage by trypsin (TRY) and chymotrypsin (CHT) are indicated with arrows. Predictions of secondary structure (α-helix [α], β-strand [β], β-turn [τ], and no prediction [•]) are indicated below each position and were determined with the program PEPTIDESTRUCTURE from the University of Wisconsin Genetics Computer Group sequence analysis package by the techniques of Chou and Fasman (8) (ch) and Garnier et al. (14) (ga). A consensus (CO) prediction is defined by matches between the two techniques. Two regions of sequence predicted to form α-helices are additionally shown in helical-wheel projections (42). In the helical wheels, charged residues (D, E, K, R) are labeled with their charges and hydrophobic residues (A, I, L, M, F) are written in larger size; residues are numbered from the first one included in each projection.

properties suggesting that they play an obligate role in permitting reoviruses to penetrate into the cytoplasm as an early event in infection of cells. Treating L cells with weak bases like ammonium chloride inhibits viral growth, apparently by blocking proteolytic cleavages that convert infecting virions to ISVPs inside cellular endosomes or lysosomes; ISVPs that have been generated by protease treatment in vitro are insensitive to this inhibition (2, 47). In addition, ISVPs, but not virions or cores, can mediate the release of ⁵¹Cr from cells to which they have been adsorbed, suggesting that ISVPs specifically are capable of penetrating into and disrupting the lipid bilayer of cell membranes (5, 28). The viral M2 gene, which encodes protein μ1, was shown recently to determine differences between reovirus strains in the capacity of their ISVPs to mediate ⁵¹Cr release (28). The μ1 protein was also shown recently to be myristoylated at its amino terminus, leading us to suggest that the capacity of ISVPs to interact with cell membranes is mediated by the μ1 protein or it proteolytic fragments, with the amino-terminal myristoyl group playing an important role (37). Given this idea, we have undertaken studies to characterize more completely the structural components of ISVPs in an effort to understand how these particles are involved in penetration.

In the present study, we demonstrated that cleavage of the μ1 and μ1C proteins during generation of ISVPs by treatment of virions with exogenous endoproteases in vitro yields not only large (~59- and 63-kDa) amino-terminal fragments (μ1δ and δ) but also a smaller (~13-kDa) carboxy-terminal

TABLE 3. Properties of reovirus T1L μ1 protein and fragments

Protein[a]	No. of residues		Net charge[d]	pI[e]
	Acidic[b]	Basic[c]		
μ1	75	64	−12	4.82
μ1N	2	1	−2	3.58
μ1C	73	63	−10	4.89
μ1δ (TRY)	67	45	−23	4.38
δ (TRY)	65	44	−21	4.42
φ (TRY)	8	19	+11	10.99
μ1δ (CHT)	67	44	−24	4.35
δ (CHT)	65	43	−23	4.39
φ (CHT)	8	20	+12	11.12

[a] The μ1δ, δ, and φ fragments are those generated by treatment of virions with either trypsin (TRY) or chymotrypsin (CHT). The values do not reflect any additional cleavages that may occur near the carboxy termini of these fragments. Deduced T1L μ1 sequence is from reference 53.

[b] Acidic residues are aspartic acid and glutamic acid.

[c] Basic residues are arginine, lysine, and histidine.

[d] Calculated after assigning each acidic residue and free carboxy terminus a value of −1 and each basic residue and free amino terminus a value of +1. The values for μ1, μ1δ, and μ1N reflect that the amino terminus of each is blocked with a myristoyl group.

[e] Calculated by using the program ISOELECTRIC from the University of Wisconsin Genetics Computer Group sequence analysis package. The values for μ1, μ1δ, and μ1N reflect that the amino terminus of each is blocked with a myristoyl group.

fragment (designated φ in this study) (Fig. 2). For cleavage with trypsin, we showed that stoichiometric quantities of both the μ1δ/δ and φ fragments are generated from μ1/μ1C and remain attached to ISVPs. These conclusions differ from the previous literature, in which the likely occurrence of the carboxy-terminal fragment was not noted and in which the carboxy-terminal region of μ1C was implied to be degraded (or at least to be lost from viral particles) at the time of generation of δ (3, 9, 20, 44). The φ protein is likely to remain attached to ISVPs by noncovalent interactions with δ, μ1N, and/or components of the viral core after its peptide backbone is separated from that of δ by cleavage. Knowing that stoichiometric amounts of φ are present in ISVPs is important for interpreting other structural studies of reovirus particles, such as a recent one involving cryoelectron microscopy (10): 600 copies of φ account for ~7.8 MDa of mass in ISVPs, and the σ3 protein is confirmed to be the only major structural element missing between ISVPs and virions (41). In addition, because φ remains in ISVPs, the carboxy-terminal portion of μ1 may play a role in specialized functions ascribed to ISVPs; for example, φ might participate directly in cytoplasmic penetration by reoviruses.

The amino terminus of φ was determined directly in this study by amino-terminal protein sequencing; however, the carboxy terminus of φ was not precisely defined (Fig. 2). Evidence is presented which indicates that chymotrypsin effects an additional cleavage somewhere near the carboxy terminus of φ. In fact, other evidence suggests that cleavage(s) near the carboxy terminus of μ1/μ1C occurs as a rapid event during treatment of virions with most or all proteases (including trypsin) so that φ may have a small peptide missing from its carboxy terminus in all cases (36). The carboxy terminus of δ also was not defined precisely in this study (Fig. 2). Since trypsin cleaves more carboxy terminal in the μ1 sequence than chymotrypsin (according to the amino-terminal sequences of the respectively generated φ fragments), the trypsin-generated δ should be slightly larger

than that generated with chymotrypsin; however, the trypsin-generated δ has a faster mobility in SDS-polyacrylamide gels than the chymotrypsin-generated one (1; data not shown), suggesting that it is smaller. This observation indicates either that there is a paradoxical difference in mobility attributable to the three-residue difference at the carboxy termini of the δ fragments or that trypsin effects an additional cleavage near the carboxy terminus of δ, which is more amino-terminal in the μ1 sequence than any additional cleavage effected at the carboxy terminus of δ by chymotrypsin. Future studies may warrant more-precise identifications of the carboxy termini of δ and φ generated with different proteases. For example, we have recently observed φ proteins generated by proteolytic cleavage of virions inside cultured cells at early times after infection (34); studies of such φ proteins generated in vivo may be useful for identifying whether particular cellular or host proteases are involved in cleaving μ1 and whether the δ and φ fragments require specific amino and carboxy termini to mediate their functions.

An interesting observation in regard to the role of φ in infection by reoviruses concerns the highly basic pI of φ and its difference from that of the other fragments of μ1 (Table 3). The remarkable difference in charge between φ and δ seems likely to have relevance. It suggests a distinction between the functions of the φ and δ portions of μ1, which might in turn explain why their peptide backbones are separated by cleavage early in infection. One possibility for the function of φ is that the excess of positively charged residues in its sequence permits it to have important electrostatic interactions with other negatively charged viral or cellular components (such as the negatively charged surfaces of cell membranes). Electrostatic interactions have been proposed to be important for the proper orientation of pore-forming toxins relative to the cell membrane before their insertion into the lipid bilayer (39).

Other interesting observations about the role of φ in penetration concern the predicted amphipathic α-helices that flank the δ-φ junction (Fig. 7). First, this type of amphipathic α-helix (having an apolar face that covers about one-half of the α-helical surface and exhibits negligible twist along the helical axis) constitutes a well-described motif for permitting peptide sequences to penetrate into the lipid bilayer of cell membranes and is widely distributed in nature (21, 22, 32, 38, 39, 43). Amphipathic α-helices are thought to be capable of partitioning half in the hydrophobic core of the lipid bilayer and half in the hydrophilic environment at the surface of the bilayer; in addition, in multimeric form, they are sometimes capable of assembling structures that span the membrane and create pores (25, 26, 38, 49). Amphipathic α-helices having charged residues arranged as in the one predicted near the carboxy terminus of δ are especially well-suited to interact with phospholipids, or other bilayer components like sphingomyelin, having zwitterionic head groups: the acidic residues are properly positioned to interact with the positively charged choline, ethanolamine, or sphingosine moieties; the basic residues to interact with the negatively charged phosphate groups; and the apolar residues to interact with the hydrophobic fatty acyl tails within the lipid bilayer (22, 43). In addition, the occurrence of lysine and arginine residues, which are themselves amphipathic, at the boundaries between polar and apolar faces of an amphipathic α-helix may promote lipid binding by providing additional hydrophobic interactions (22). Given these facts, it seems reasonable to postulate that amphipathic α-helices in

μ1 might be important for its interactions with a cell membrane during penetration by reoviruses.

In fact, amphipathic α-helices have been implicated in penetration of cell membranes by other animal viruses. The fusion proteins of many enveloped viruses undergo cleavage by exogenous proteases, generating carboxy-terminal fragments that have a region of hydrophobic sequences near their amino termini (recently reviewed in reference 52). These sequences are called fusion peptides because they appear to mediate fusion between viral and cellular membranes during penetration; examples are found in the hemagglutinin protein of orthomyxoviruses and the envelope protein of retroviruses. Importantly for the current discussion, these fusion peptides are thought to interact with the cell membrane in the form of amphipathic α-helices (16, 25, 52). Recently, sequences at the amino terminus of capsid protein VP1 of polioviruses (members of the picornavirus family of nonenveloped viruses) were proposed to play an essential role in penetration of cell membranes by these viruses and to effect this function also in the form of an amphipathic α-helix (12, 24). Thus, amphipathic α-helices may be common to the mechanisms of penetration by both enveloped and nonenveloped viruses, the latter including the mammalian reoviruses according to the present study. The proximity of these α-helices to a protein terminus generated by endoproteolytic cleavage in most instances may facilitate membrane binding by reducing conformational and/or topological constraints and may provide a means for regulating this activity (decreased binding capacity before cleavage).

For penetration by reoviruses, the μ1 protein might be expected to disrupt the membrane of the endosome or lysosome grossly to permit the entry of a viral particle into the cytoplasm (similar to the mechanism proposed for adenoviruses [40]), or it might introduce the viral particle (or a portion of it) across the membrane by a more precise, less disruptive mechanism. We previously proposed a role for the myristoylated μ1N peptide in this process and now suggest that the newly identified amphipathic α-helices in μ1 (including one near the amino terminus of φ) are likely to act in concert with the myristoyl groups during penetration. These suggestions are similar to ones recently made in regard to penetration by picornaviruses (12). Current studies are aimed at providing a molecular description of the role of μ1 and its fragments in penetration by reoviruses.

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