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Mammalian Reoviruses Contain a Myristoylated Structural Protein

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The structural protein μ1 of mammalian reoviruses was noted to have a potential N-myristoylation sequence at the amino terminus of its deduced amino acid sequence. Virions labeled with [^{3}H]myristic acid were used to demonstrate that μ1 is modified by an amide-linked myristoyl group. A myristoylated peptide having a relative molecular weight (M_r) of ~4,000 was also shown to be a structural component of virions and was concluded to represent the 4.2-kDa amino-terminal fragment of μ1 which is generated by the same proteolytic cleavage that yields the carboxy-terminal fragment and major outer capsid protein μ1C. The myristoylated 4,000-M_r peptide was found to be present in reovirus intermediate subviral particles but to be absent from cores, indicating that it is a component of the outer capsid. A distinct large myristoylated fragment of the intact μ1 protein was also identified in intermediate subviral particles, but no myristoylated μ-region proteins were identified in cores, consistent with the location of μ1 in the outer capsid. Similarities between amino-terminal regions of the reovirus μ1 protein and the poliovirus capsid polyprotein were noted. By analogy with other viruses that contain N-myristoylated structural proteins (particularly picornaviruses), we suggest that the myristoyl group attached to μ1 and its amino-terminal fragments has an essential role in the assembly and structure of the reovirus outer capsid and in the process of reovirus entry into cells.

Members of four different animal virus families—retroviruses (12, 21), papovaviruses (41, 49), picornaviruses (8, 33), and hepadnaviruses (34)—have been shown to contain a virion structural protein that is modified by amino-terminal acylation with the 14-carbon saturated fatty acid myristic (*n*-tetradecanoic) acid. This report suggests that the mammalian reoviruses represent a fifth animal virus family whose members contain an N-myristoylated structural protein.

The mammalian and avian reoviruses, along with rotaviruses and orbiviruses, constitute the animal virus members of the family *Reoviridae* (31). The genome of reoviruses consists of 10 segments of linear double-stranded RNA. Reovirus virions appear to have icosahedral symmetry and do not contain a lipid envelope. The virions are built from two protein capsid layers which include a core that encloses the viral gene segments and contains the viral transcriptase and an outer capsid that interacts with cells at early stages of the viral replication cycle (reviewed in references 39 and 53).

The outer capsid layer of reovirus virions is largely formed from viral proteins μ1C and σ3, which are present in approximately equal numbers (23). The μ1C protein was found in pulse-chase experiments to arise posttranslationally from the slightly larger μ1 protein (57), which is the primary translation product of the reovirus M2 gene segment (29, 32). A small amount of uncleaved μ1 protein is also routinely seen in purified virions and has been suggested to be a component of either the viral core (48) or the outer capsid (23, 32). Amino-terminal sequence analysis of μ1C protein obtained from purified virions (23, 35) identified its amino-terminal residue as proline 43 in the deduced μ1 amino acid sequence (23, 56), consistent with the proposal that μ1C is derived from μ1 via a proteolytic cleavage. The molecular masses of the μ1 and μ1C proteins were calculated from their deduced amino acid sequences as 76.3 and 72.1 kDa, respectively (23). Virion-associated μ1C protein (and perhaps μ1 protein also) can undergo a distinct cleavage nearer its carboxy terminus (23, 32a) after exposure to exogenous proteases in vitro (2, 24, 45). A similar cleavage occurs at early times following infection of cells in culture (5, 7, 46, 50) and in the lumen of the mouse small intestine (1) and may have important biologic consequences. Genetic studies have implicated a role for M2 and its protein products in several properties of reovirus: response to protease treatment and transcriptase activation in vitro (13), response to treatment with ethanol and phenol in vitro (14, 54a), induction of tolerance to reovirus-specific antigens after oral inoculation in adult mice (38), neurovirulence after intracranial inoculation in newborn mice (22), and capacity of reovirus intermediate subviral particles (ISVPs) to interact with cell membranes (26a).

To gain some understanding of the molecular mechanisms of the M2-associated properties of reovirus, we began studies to characterize the biochemical and structural features of the μ1 protein and its proteolytic cleavage fragments. In this study, we identified a potential N-myristoylation sequence at the amino terminus of the deduced μ1 amino acid sequence and showed that μ1, but not μ1C, is modified with an amide-linked myristoyl group. We identified a myristoylated 4,000-M_r peptide that is also found in virions and conclude that it represents the 4.2-kDa amino-terminal fragment of μ1 which is generated by the same proteolytic cleavage that yields the carboxy-terminal fragment μ1C. We suggest the name μ1N for this peptide. In studies involving reovirus subviral particles, we showed that the myristoylated μ1N peptide and a large myristoylated fragment (named μ1δ) that is generated from intact μ1 protein by chymotrypsin treatment are both present in ISVPs but absent from cores, suggesting that they are components of the viral outer capsid. Similarities between amino-terminal regions of μ1 and the poliovirus capsid polyprotein were noted. These findings have led us to suggest that the myristoyl group attached to μ1 and its amino-terminal fragments plays an

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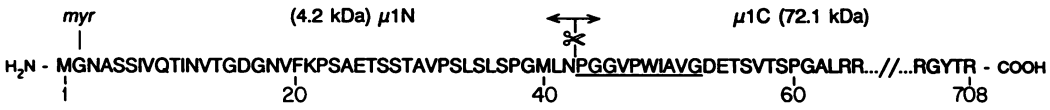

FIG. 1. Partial amino acid sequence of reovirus T3D μ1 protein. A potential N-myristoylation sequence occurs at the amino terminus of μ1. The outer capsid protein μ1C (predicted mass, 72.1 kDa) is generated from μ1 by proteolytic cleavage (scissors). The amino-terminal sequence of μ1C (underlined) was reported by Jayasuriya et al. (23). A peptide comprising μ1 residues 2 to 42 plus an amino-terminal myristoyl group would have a predicted mass of 4.2 kDa and might be generated by the same cleavage that generates μ1C. We suggest that this putative peptide be called μ1N. *myr*, Myristoyl group.

important role in the assembly and structure of the reovirus outer capsid and in the process of reovirus entry into cells.

MATERIALS AND METHODS

Cells, viruses, and preparation of purified virions. Mouse L cells were grown in suspension in Joklik's modified minimal medium (Irvine Scientific, Irvine, Calif.) supplemented to contain 5% fetal bovine serum and 2 mM glutamine. Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) were the viruses primarily used in this study. Purified reovirus virions were obtained after extraction from infected cells and CsCl density gradient centrifugation as described previously (17). Virion storage buffer consisted of 150 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris hydrochloride (pH 7.5). Virion particle concentrations were determined by measuring the OD_{260} and using the conversion factor given by Smith et al. (48). To obtain purified virions containing [^{35}S]methionine- and [^{35}S]cysteine-labeled proteins, Tran^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, Calif.) (5 to 12.5 μCi/ml) was added to the cell suspension at the initiation of infection. To obtain purified virions containing [^{3}H]myristate-labeled proteins, [^{3}H]myristic acid (12.5 μCi/ml; NEN Research Products, Boston, Mass.) was added to the cell suspension at the initiation of infection. All infections were allowed to continue for 60 to 84 h before cultures were harvested for virion purification.

SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (26). Gradient gels were prepared by the method of Hames (19). SDS-urea-polyacrylamide gels for the analysis of low-molecular-weight proteins were prepared by the method of Hashimoto et al. (20). In this technique, the resolving gel included an acrylamide gradient (10 to 18% or 5 to 18%), an increased concentration of bisacrylamide (5%), and 7 M urea, and the stacking gel was prepared by the method of Laemmli (26). In all experiments, sample buffer contained final concentrations (after the addition of virus sample) of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue. Samples were disrupted by heating in a boiling-water bath for 1 to 2 min. Gels containing radiolabeled proteins were treated with Enlightning (New England Nuclear Corp., Boston, Mass.), dried under vacuum, and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. Coomassie-stained gels were dried between cellophane (Bio-Rad Laboratories, Richmond, Calif.). Estimates of protein molecular weight in SDS-polyacrylamide gels were determined by comparisons with molecular weight marker proteins (Bethesda Research Laboratories, Gaithersburg, Md.).

ISVPs and cores. ISVPs and cores were obtained by treating purified virions in vitro with 200 μg of TLCK-treated α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) per ml. All such treatments were performed in virion storage buffer at 37°C. To obtain ISVPs, T1L virions at a concentration of 1×10^{13} particles per ml or T3D virions at a concentration of 2×10^{12} particles per ml were treated for the specified times. To obtain cores, T1L virions at a concentration of 3.5×10^{13} particles per ml or T3D virions at a concentration of 1×10^{13} particles per ml were treated for the specified times. Chymotrypsin treatments were ended by the addition of 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and placement of the samples at 4°C. ISVPs and cores were isolated from the samples by CsCl density gradient sedimentation as described previously (17), except that centrifugation was performed with a Beckman 28.1 rotor spun at 26,000 rpm for 2 to 4 h. After dialysis in virion storage buffer, the isolated particles were kept at 4°C until use.

Acid hydrolysis and thin-layer chromatography. Samples of [^{3}H]myristate-labeled T3D virions were subjected to acid hydrolysis for analysis by thin-layer chromatography. Samples (10 μl, ~90,000 cpm) in virion storage buffer were diluted 1:20 with 6 N HCl and heated at 110°C in a reflux tube for 14 h. The hydrolysates were extracted twice with 100 μl of toluene. Toluene fractions were pooled, lyophilized, and resuspended in 20 μl of toluene. SDS-polyacrylamide gel slices containing μ1 and μ1N polypeptides from an equivalent number of [^{3}H]myristate-labeled T3D virions were placed in 200 μl of 6 N HCl and subjected to hydrolysis and extraction in the same fashion as virions. For each type of sample, 4- to 5-μl volumes containing approximately equal counts of ^{3}H were applied with drawn-tip micropipettes to Si-C_{19} reversed-phase thin-layer chromatography plates (10 by 10 cm; J. T. Baker Chemical Co., Phillipsburg, N.J.). Samples of toluene solutions containing [^{3}H]myristic acid (NEN Research Products), [^{3}H]palmitic acid (Amersham Corp., Arlington Heights, Ill.), and nonradiolabeled lauric, myristic, palmitic, and stearic acids (Aldrich Chemical Co., Milwaukee, Wis.) were also applied to the plates as markers. Chromatograms were developed with acetonitrile-acetic acid (1:1). The positions of nonradiolabeled compounds were visualized by incubation of the developed chromatogram in an iodine chamber (47). The positions of ^{3}H-labeled compounds were visualized by fluorography after the developed chromatogram was sprayed with En^{3}Hance (NEN Research Products).

RESULTS

μ1 protein contains a potential N-myristoylation sequence. Examination of the deduced μ1 amino acid sequence of reovirus strains T1L, type 2 Jones, and T3D (23, 56) revealed conserved amino-terminal sequences that are similar to those of other viral and cellular N-myristoylated proteins (42, 51). Specific features of the μ1 sequence that are characteristic of those proteins are a glycine residue in position 2 and a small uncharged residue in position 6 (serine, threonine, alanine, or glycine in known N-myristoy-

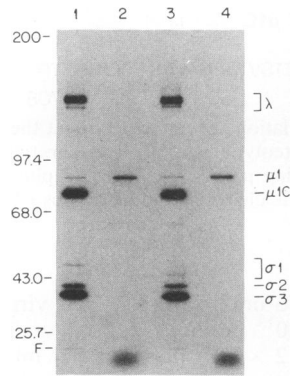

FIG. 2. SDS-polyacrylamide gel of radiolabeled proteins from purified reovirus virions. Purified virions of ^{35}S-labeled (lanes 1 and 3) and [^{3}H]myristate-labeled (lanes 2 and 4) reovirus strains T1L (lanes 1 and 2) and T3D (lanes 3 and 4) were disrupted by boiling in sample buffer and subjected to electrophoresis in an SDS-polyacrylamide gel (8.25% acrylamide, 2.6% bisacrylamide). Proteins were visualized by fluorography. Molecular weight marker proteins were included in the gel, and their positions are indicated (10^3 M_r) on the left. The position of the ion front (F) is also indicated on the left. Virion proteins are identified on the right.

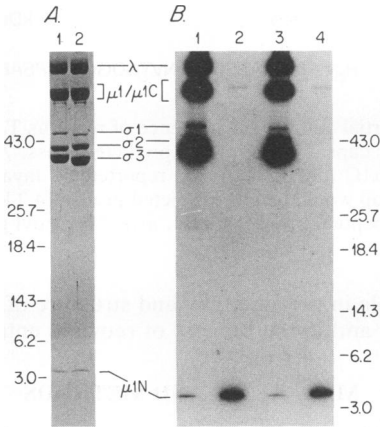

FIG. 3. SDS-urea-polyacrylamide gradient gels of nonradiolabeled and radiolabeled proteins from purified reovirus virions. (A) Equal quantities (4×10^{11} particles) of nonradiolabeled virions of reovirus strains T1L (lane 1) and T3D (lane 2) were disrupted by boiling in sample buffer and subjected to electrophoresis in an SDS-urea-polyacrylamide gradient gel (10 to 18% acrylamide, 5% bisacrylamide). Proteins were visualized by Coomassie blue staining. (B) Purified virions of ^{35}S-labeled (lanes 1 and 3) and [^{3}H] myristate-labeled (lanes 2 and 4) reovirus strains T1L (lanes 1 and 2) and T3D (lanes 3 and 4) were disrupted by boiling in sample buffer and subjected to electrophoresis in an SDS-urea-polyacrylamide gradient gel (10 to 18% acrylamide, 5% bisacrylamide). Proteins were visualized by fluorography. Molecular weight marker proteins were included in both gel A and gel B, and their positions are indicated (10^3 M_r) on the left and right, respectively. Virion proteins, including the ~4,000-M_r μ1N peptide, are identified in the space between the gels.

lated proteins; serine in μ1) Fig. 1). In the N-myristoylated proteins, a glycine residue occurs at the protein amino terminus subsequent to the proteolytic removal of the initiator methionine residue (in most cases) or a leader peptide (8, 51). The myristoyl group is joined to this amino-terminal glycine via an amide bond (21). The currently available data do not permit an unambiguous myristoylation consensus sequence to be defined. Thus, the N-myristoylation of μ1 cannot be predicted with certainty from its amino acid sequence alone.

Radioactivity from [^{3}H]myristic acid is incorporated into μ1. To determine whether the amino-terminal sequence of μ1 can direct its myristoylation, we grew reovirus strains T1L and T3D in suspension cultures of mouse L cells to which [^{3}H]myristic acid was added early in infection. Preparations of purified virions that were obtained from these cultures were found to contain a large amount of associated radioactivity that was not removed by extensive dialysis. The radioactivity in the dialyzed preparations was determined to be incorporated into virion particles by its comigration with ^{35}S-labeled virions after both CsCl gradient density sedimentation and sucrose gradient velocity sedimentation (data not shown). When the [^{3}H]myristate-labeled T1L and T3D virions were analyzed by SDS-PAGE (Fig. 2, lanes 2 and 4), a radiolabeled protein was observed in each which comigrated with the μ1 protein seen in samples of ^{35}S-labeled virions (Fig. 2, lanes 1 and 3). Similar amounts of ^{3}H were not associated with μ1C or any other of the known reovirus structural proteins. Because μ1C is generated by the proteolytic removal of a small region of sequence from the amino terminus of μ1, including the potential N-myristoylation sequence (Fig. 1), the lack of [^{3}H]myristate labeling of μ1C was predictable. Similarly, the lack of [^{3}H] myristate labeling of other viral structural proteins was predictable, since an examination of deduced amino acid sequences revealed that no other reovirus T3D protein has a potential myristoylation sequence at its amino terminus. Thus, the results of this experiment suggest that ^{3}H from [^{3}H]myristic acid was incorporated into the reovirus μ1 protein in a specific fashion.

Purified virions contain a 4,000-M_r peptide that is radiolabeled with [^{3}H]myristic acid. The proteolytic processing event that generates μ1C from μ1 might also yield a small peptide that corresponds to the amino terminus of μ1 and includes the potential N-myristoylation sequence (Fig. 1). Smith et al. (48) identified a ~8,000-M_r peptide (component viii) in purified T3D virions and top component particles (but not cores) which they proposed to be the amino-terminal fragment of μ1. Little subsequent work has addressed these findings. The molecular mass of the amino-terminal peptide predicted from the μ1 amino acid sequence is ~4.2 kDa (23); removal of the initiator methionine and addition of a myristoyl group to glycine 2 would not change its predicted mass. We have adopted the name μ1N for this proposed amino-terminal fragment of μ1. Similarly, we suggest that the name μ1C can be used to reflect that it is the carboxy-terminal fragment of μ1 (rather than simply a cleaved form of μ1 according to current usage).

In an attempt to determine whether virions contain μ1N, we used SDS-urea-polyacrylamide gels that are designed to resolve small protein species (20). When purified virions of reovirus strains T1L and T3D were analyzed in this gel system, a peptide of ~4,000 M_r was visualized by Coomassie blue staining (Fig. 3A). Our ability to detect this peptide with Coomassie blue suggested that it occurs in virion preparations in high copy number. All purified virion preparations of T1L and T3D as well as those of type 2 Jones, type 3 Abney, and five other type 3 reovirus strains were found to contain similar quantities of a 4,000-M_r peptide (data not shown), suggesting that it is a distinct structural component of all mammalian reoviruses. Because of its size and quantity, this peptide seemed likely to represent μ1N.

We reasoned that if the 4,000-M_r peptide were μ1N, it should be myristoylated, given that μ1N is expected to contain the myristoylation sequence of μ1 at its amino terminus. To test this hypothesis, we first examined the proteins from ^{35}S-labeled T1L and T3D virions in the gel system described by Hashimoto et al. (20). Radioactivity was found to be associated with the 4,000-M_r peptide but to constitute only a minor amount of that present in all the virion structural proteins (Fig. 3B, lanes 1 and 3). The observation that the peptide was labeled with ^{35}S was not inconsistent with its identification as μ1N, since μ1N is expected to contain a single internal methionine residue (methionine 40) (Fig. 1). On the other hand, when T1L and T3D virions that had been grown in the presence of [^{3}H]myristic acid were examined, the 4,000-M_r peptide was found to have large amounts of associated radioactivity (Fig. 3B, lanes 2 and 4). Radioactivity was also found associated with the μ1 protein in these virions, but the amount was minor compared with that in the peptide. The latter finding correlates with the smaller quantities of μ1 than μ1C seen in virions, and since μ1C and μ1N are likely to be present in virions in equal copy number, it is consistent with the identification of the 4,000-M_r peptide as μ1N. On the basis of these results, we conclude that the μ1N peptide is present in virions and that it is myristoylated.

μ1 and the 4,000-M_r μ1N peptide are modified by an amide-linked myristoyl group. The data presented indicate that μ1 and the 4,000-M_r μ1N peptide were radiolabeled in virions grown in [^{3}H]myristic acid; however, they do not indicate whether the radiolabel was still associated with a myristoyl group in these modified proteins. Interconversions between myristic and palmitic (*n*-hexadecanoic) acid, for example, have been reported to occur during long periods of metabolic labeling (43) such as those used to obtain ^{3}H-labeled virions in this study. Palmitoylation of eukaryotic proteins, including a number of viral structural proteins, is known to occur by a different mechanism than myristoylation; a palmitoyl group is attached to proteins via a thioester bond, whereas a myristoyl group is attached via an amide bond (reviewed in reference 44).

To identify the type of bond that joins the ^{3}H-labeled moiety to the reovirus μ1 and μ1N proteins, [^{3}H]myristate-labeled T3D virions were disrupted by boiling in 1% SDS and subjected to treatments designed to detach the radiolabeled moiety. First, samples of disrupted virions or free [^{3}H]myristic acid were directly extracted with diethyl ether. Radioactivity in the virion sample remained in the aqueous phase after extraction, while that in the free [^{3}H]myristic acid control sample was extracted into the organic phase (data not shown), confirming that the ^{3}H-labeled moiety was covalently attached to virion proteins. Next, samples of disrupted virions or free [^{3}H]myristic acid were treated with 0.2 M KOH in methanol (40) before being lyophilized, neutralized, and extracted with diethyl ether. Again, radioactivity in the virion sample remained in the aqueous phase after extraction, while that in the free [^{3}H]myristic acid sample was extracted into the organic phase (data not shown). Since methanolic KOH is expected to cleave ester and thioester but not amide bonds (8), these findings suggest that the ^{3}H-labeled moiety was attached to reovirus proteins via an amide bond.

To identify the ^{3}H-labeled moiety itself, [^{3}H]myristate-labeled T3D virions were incubated in 6 N HCl in order to hydrolyze the virion proteins and then were examined by reversed-phase thin-layer chromatography (8, 49). The μ1 and μ1N peptide bands excised from polyacrylamide gels of

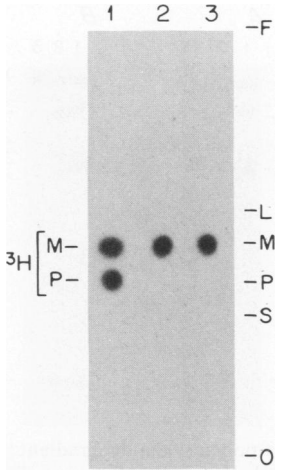

FIG. 4. Reversed-phase thin-layer chromatogram of radiolabeled fatty acids from purified reovirus virions. The following samples were applied to a reversed-phase C_{18} thin-layer chromatography plate: [^{3}H]myristic (M) and [^{3}H]palmitic (P) acids (positions labeled at left) and a mixture of these compounds (lane 1); acid-hydrolyzed [^{3}H]myristate-labeled T3D virions (lane 2); μ1 (not shown) and μ1N (lane 3) polypeptides that were acid hydrolyzed in SDS-polyacrylamide gel slices obtained from samples of [^{3}H]myristate-labeled T3D virions; and nonradiolabeled lauric (L), myristic (M), palmitic (P), and stearic (S) acids (positions labeled at right). The chromatogram was developed with acetonitrile-acetic acid (1:1). Nonradiolabeled compounds were visualized by incubation of the developed chromatogram in an iodine chamber. Radiolabeled compounds were visualized by fluorography. F, solvent front; O, origin.

[^{3}H]myristate-labeled T3D virions were also subjected to acid hydrolysis and analyzed in the same manner. [^{3}H]myristic acid and [^{3}H]palmitic acid (Fig. 4, lane 1) and nonradiolabeled lauric (*n*-dodecanoic) acid, myristic acid, palmitic acid, and stearic (*n*-octadecanoic) acid (Fig. 4, positions indicated at right) were included as markers in the chromatogram. Analysis of the virion hydrolysis extract revealed a ^{3}H-labeled compound which comigrated with the [^{3}H]myristic acid and nonradiolabeled myristic acid markers (Fig. 4, lane 2). The ^{3}H-labeled compound released from both the gel-purified μ1 (data not shown) and μ1N (Fig. 4, lane 3) proteins comigrated with the myristic acid markers as well. These findings indicate that [^{3}H]myristic acid was incorporated into both μ1 and μ1N as a myristoyl group.

ISVPs contain myristoylated proteins but cores do not. Two distinct types of reovirus subviral particles, ISVPs and cores, are thought to be generated as functional intermediates at early times after reovirus infection of cells (5, 7, 46, 50). Each of these subviral particles can also be generated by treatment of virions with chymotrypsin in vitro (2, 24, 45). ISVPs differ from virions in that they lack the major outer capsid protein σ3 and contain an endoproteolytically cleaved form of the other major outer capsid protein μ1C (2, 7, 24, 32a, 45, 46, 50). They are generally infectious and may represent the virus form that is able to effect a direct interaction with cell membranes during penetration of reovirus into the cell cytoplasm (3, 5). Cores, on the other hand, lack σ3 as well as μ1C (and its cleavage products) and σ1. They have greatly reduced infectivity and are capable of generating full-length viral mRNA transcripts when incubated under the appropriate conditions (24, 48). How cores are generated in vivo and how they are introduced into the cell cytoplasm are currently poorly understood. Thus, an

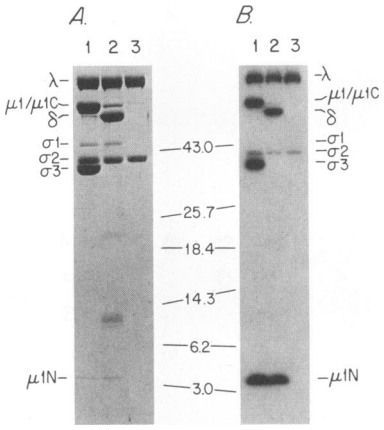

FIG. 5. SDS-urea-polyacrylamide gradient gels of nonradiolabeled and radiolabeled proteins from gradient-purified virions, ISVPs, and cores. (A) Nonradiolabeled purified virions of reovirus T1L were treated with chymotrypsin for 60 min in order to generate either ISVPs or cores. The subviral particles were then isolated by CsCl density gradient sedimentation. Approximately equal quantities (4×10^{11} particles) of the virions (lane 1), ISVPs (lane 2), and cores (lane 3) were disrupted by boiling in sample buffer and subjected to electrophoresis in an SDS-urea-polyacrylamide gradient gel (10 to 18% acrylamide, 5% bisacrylamide). Proteins were visualized by Coomassie blue staining. Virion proteins are indicated on the left. The ~13,000-M_r protein band visible in lane 2 is known to be the carboxy-terminal fragment of μ1C that is generated along with the amino-terminal fragment δ by chymotrypsin cleavage (32a). (B) Purified virions of reovirus T3D that were radiolabeled with either [^{3}H]myristic acid or ^{35}S were mixed together and treated with chymotrypsin for 60 min in order to generate either ISVPs or cores. Viral particles were then isolated from each sample by CsCl density gradient centrifugation. The mixedly radiolabeled virions (lane 1), ISVPs (lane 2), and cores (lane 3) were disrupted by boiling in sample buffer and subjected to electrophoresis in an SDS-urea-polyacrylamide gradient gel (10 to 18% acrylamide, 5% bisacrylamide). Proteins were visualized by fluorography. Virion proteins are indicated on the right. Molecular weight marker proteins were included in both gel A and gel B, and their positions are indicated (10^3 M_r) in the space between the gels.

accurate assessment of the viral proteins present in both ISVPs and cores seems necessary for an understanding of early events in reovirus infection.

We performed experiments to determine whether reovirus subviral particles contain the 4,000-M_r μ1N peptide. ISVPs and cores were initially prepared by in vitro chymotrypsin treatment of nonradiolabeled T1L virions. The subviral particles were isolated by sedimentation in CsCl density gradients and examined for low-molecular-weight protein components in SDS-urea-polyacrylamide gels (Fig. 5A). A 4,000-M_r peptide which comigrated with the peptide from virions was found to be present in ISVPs but absent from cores; thus, ISVPs but not cores appear to contain μ1N. To confirm this finding, we mixed T3D virions that were labeled with either [^{3}H]myristic acid or ^{35}S and treated them with chymotrypsin in order to generate either ISVPs or cores. The ^{35}S-labeled virions were included in these samples to serve as markers for the locations of viral particles in CsCl gradients. The particle-containing fractions in these gradients were identified by scintillation counting (data not shown). ^{35}S-labeled and [^{3}H]myristate-labeled virions from a mock treatment sample containing virions but no chymotrypsin were found to cosediment. Similarly, ^{3}H from the ISVP treatment sample was found to cosediment with the ^{35}S-labeled ISVPs, suggesting that ISVPs contain [^{3}H]myristate-labeled proteins. On the other hand, ^{3}H from the core treatment sample was found not to cosediment with the ^{35}S-labeled cores, suggesting that cores do not contain [^{3}H]myristate-labeled proteins; the ^{3}H in the core treatment sample instead sedimented in a distinct peak near the top of the CsCl gradient. The particle-containing fractions from each gradient were separately pooled and examined in SDS-urea-polyacrylamide gels (Fig. 5B). Virions and ISVPs were found to contain similar quantities of the 4,000-M_r μ1N peptide; however, no μ1N was detectable in cores. The amount of μ1N in ISVPs relative to that in virions was estimated by first quantitating the fluorographic intensities of λ and μ1N protein bands in multiple gel lanes and then standardizing the values obtained for the μ1N band relative to the values obtained for the λ band for each particle type. The results of this analysis (data not shown) suggest that virions and ISVPs contain equivalent amounts of μ1N.

We also performed experiments to determine what happens to the small amount of intact μ1 protein when virions are converted to ISVPs. ^{35}S-labeled T1L virions were treated with chymotrypsin in order to generate ISVPs. A rapid degradation of σ3 and a slower cleavage of μ1C to yield its large amino-terminal fragment δ (23) were observed after SDS-PAGE (Fig. 6A, lanes 1 to 3). The small amount of intact μ1 protein in virions has also been suggested to be cleaved during this treatment to yield a proportionally sized δ fragment (named μ1δ by Jayasuriya et al. [23] and δ_1 by Ewing et al. [16]) (Fig. 6A, lanes 2 and 3). To test this suggestion, we treated [^{3}H]myristate-labeled T1L virions with chymotrypsin to generate ISVPs (Fig. 6A, lanes 4 to 6). [^{3}H]myristate-labeled μ1 was converted to a faster-migrating protein, which comigrated with the proposed μ1δ fragment from ^{35}S-labeled ISVPs. These findings indicate that a δ cleavage fragment is generated from both μ1 and μ1C during the generation of ISVPs by chymotrypsin.

As a final experiment in this study, we used [^{3}H]myristate-labeled virions to address whether reovirus cores contain μ1 or any large μ1-derived fragments. ^{35}S-labeled T3D virions were treated with chymotrypsin in order to generate cores, and the samples were analyzed by SDS-PAGE (Fig. 6B, lanes 1 to 4). The σ3, μ1, μ1C, μ1δ, and δ proteins appeared to be absent at the end of this treatment, although μ1δ and δ were transiently generated prior to degradation. A small amount of protein remained in the μ region of the final core sample but did not comigrate with either μ1 or the μ1δ fragment; this protein has been suggested previously to represent either the μ1 protein (48) or the μ2 protein (23, 32). To test whether this protein includes amino-terminal sequences from μ1, we treated [^{3}H]myristate-labeled T3D virions with chymotrypsin in order to generate cores (Fig. 6B, lanes 5 to 8). [^{3}H]myristate-labeled μ1δ fragment was transiently generated during the course of treatment; however, no [^{3}H]myristate-labeled μ proteins were seen in the final core sample. These findings indicate that the myristoylated μ1 and μ1δ proteins are absent from cores and are therefore components of the viral outer capsid.

DISCUSSION

In this report we demonstrate that the structural protein μ1 of mammalian reoviruses is modified by an amide-linked myristoyl group and is a component of the viral outer capsid. We also show that a myristoylated 4,000-M_r peptide is present in the outer capsid and conclude that this peptide

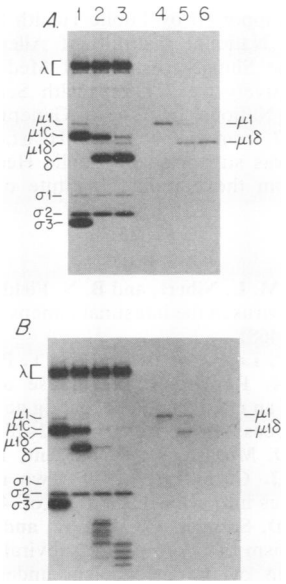

FIG. 6. SDS-polyacrylamide gels of radiolabeled proteins in ISVP and core treatment samples. (A) Purified virions of ^{35}S-labeled (lanes 1 to 3) and [^{3}H]myristate-labeled (lanes 4 to 6) T1L were each treated with chymotrypsin to generate ISVPs. Samples were taken after different times of treatment (0 min, lanes 1 and 4; 3 min, lanes 2 and 5; and 30 min, lanes 3 and 6), disrupted by boiling in sample buffer, and subjected to electrophoresis in an SDS-polyacrylamide gradient gel (5 to 10% acrylamide, 2.6% bisacrylamide). (B) Purified virions of ^{35}S-labeled (lanes 1 to 4) and [^{3}H]myristate-labeled (lanes 4 to 8) T3D were each treated with chymotrypsin to generate cores. Samples were taken after different times of treatment (0 min, lanes 1 and 5; 3 min, lanes 2 and 6; 10 min, lanes 3 and 7; and 30 min, lanes 4 and 8), disrupted by boiling in sample buffer, and subjected to electrophoresis in an SDS-polyacrylamide gradient gel (5 to 15% acrylamide, 2.6% bisacrylamide). Proteins in both gel A and gel B were visualized by fluorography. Virion proteins are identified on the left of each gel. Degradation fragments of μ1C/δ that occur transiently during chymotrypsin treatments that generate cores are seen in gel B, lanes 2 and 3.

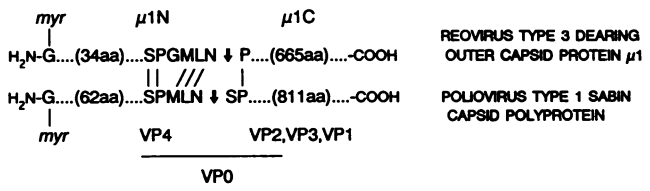

FIG. 7. Characteristics shared by capsid proteins of mammalian reoviruses and polioviruses. The reovirus outer capsid protein μ1 and the poliovirus capsid polyprotein each includes a small myristoylated peptide that is liberated from the amino terminus of the complete protein by proteolytic cleavage. The two proteins have a limited amount of sequence similarity (shown by lines connecting identical amino acid residues) around their cleavage sites (arrows). Cleavage occurs at the carboxy-terminal side of an asparagine (N) residue in each. VP0 represents poliovirus capsid proteins VP4 and VP2 that have not been separated by the indicated cleavage. The poliovirus type 1 Sabin sequence is from Toyoda et al. (52). aa, Amino acids; *myr*, myristoyl group.

represents the myristoylated 4.2-kDa amino-terminal fragment of μ1 that is generated by the same proteolytic cleavage that yields the major outer capsid protein μ1C. We suggest that the name μ1N be used for this amino-terminal fragment. The name μ1C can then be used to reflect that it is the carboxy-terminal fragment of μ1 (rather than simply a cleaved form of μ1 according to current usage). Although it is possible that the myristoyl group in μ1 and μ1N is attached to the ε-amino group of lysine 21, the similarity of the amino-terminal sequence of μ1 to those of other N-myristoylated proteins makes it likely that this group is attached to the amino-terminal amino group that is generated on glycine 2 of μ1 by removal of the initiator methionine residue. Thus, μ1 and μ1N are apt to be N-myristoylated polypeptides.

Mammalian reoviruses represent the fifth family of animal viruses (*Reoviridae*, in addition to *Retroviridae*, *Papovaviridae*, *Picornaviridae*, and *Hepadnaviridae*) that includes members containing an N-myristoylated structural protein. Rotaviruses, which are also members of the family *Reoviridae*, have been found to encode proteins (VP2 and VP6) that can be myristoylated (9); however, that observation appears to require confirmation, since the rotavirus proteins lack the amino-terminal sequences generally associated with N-myristoylation (15, 30).

The N-myristoylation of viral structural proteins has been proposed to play an important role in the assembly of other viruses. Myristoylation-defective mutants of picornaviruses (25, 27, 28) and retroviruses (6, 10, 18, 36, 37, 54) consistently fail to assemble infectious virions. Improper assembly may relate to the failure of the nonmyristoylated capsid proteins to interact appropriately with cell membranes (6, 36, 37, 54) or to the failure of the nonmyristoylated capsid proteins to be appropriately processed by viral or cellular proteases (6, 18, 25, 27, 37, 54).

The importance of myristoyl groups in the final structure of picornaviruses was revealed by X-ray diffraction analysis (8). Myristoyl groups attached to the 60 copies of capsid protein VP4 occupy positions about sites of fivefold symmetry at the icosahedral vertices of the picornavirus capsid; there they interact with one another as well as with amino acid residues from VP4 and VP3. These interactions are thought to provide essential forces stabilizing the icosahedral capsid structure. Thus, N-myristoylation of viral structural proteins may be essential to the assembly of infectious virions because myristoyl groups are important structure-determining components of the virus capsid.

Myristoyl groups attached to μ1 and μ1N are likely to be significant structural components of the reovirus outer capsid as well. We favor a model in which intact μ1 protein molecules occupy capsid positions similar to those of μ1 molecules that have been cleaved to yield μ1N and μ1C. According to this model and recent studies with freeze-dried viral particles (14a), approximately 600 copies of μ1 or its cleavage fragments are found in the reovirus outer capsid and determine the icosahedral symmetry of this capsid layer. The available data suggest that most or all copies of virion-associated μ1 and μ1N polypeptides are N-myristoylated, in which case as many as 600 myristoyl groups are found in the reovirus outer capsid. The presence of such a large number of fatty acyl chains in the outer capsid of a nonenveloped virus seems likely to have important structural and/or functional consequences.

Similarities between the outer capsid protein μ1 of reoviruses and the capsid polyprotein of polioviruses are suggested in Fig. 7. Both the reovirus outer capsid and the poliovirus capsid contain a small myristoylated amino-terminal peptide (μ1N and VP4, respectively) which is gener-

ated by proteolytic cleavage of a larger myristoylated capsid protein precursor (μ1 and VP0, respectively). This cleavage does not occur with 100% efficiency in either virus, since small amounts of precursor protein (μ1 and VP0) are identified by SDS-PAGE in purified virions of each. In addition, limited sequence similarity is found around the cleavage sites that generate the myristoylated amino-terminal peptides in the two viruses. The sequence similarity includes an asparagine residue which immediately precedes the cleavage site in each. Given that this cleavage is an autocatalytic one in picornaviruses (37a), the illustrated sequence similarity may suggest that the cleavage that generates μ1N and μ1C from μ1 in the reovirus outer capsid is also autocatalytic. On the other hand, the reovirus outer capsid and the picornavirus capsid are likely to differ in several respects. In particular, the large number of myristoyl groups in the reovirus outer capsid are unlikely to occur about the sites of fivefold symmetry at the icosahedral vertices, since those positions are limited in number and are substituted by the λ2 protein (55). If they indeed occur near symmetry axes, myristoyl groups attached to μ1N are more likely to be found about the numerous sites of threefold or sixfold symmetry predicted in the reovirus outer capsid; thus, studies with reoviruses may provide insight into the versatility that can be exhibited by myristoyl groups in the structure of virus capsids.

The myristoyl groups in polioviruses have also been proposed to have an essential function in the penetration of virions into the cytoplasm at early times of infection (8, 28, 33). The same may be true for reoviruses. Prior work (3, 5) suggested that reovirus ISVPs are capable of interacting directly with cell membranes. Recent work in our laboratory has extended this work and shown that this property is influenced by the μ1-encoding gene M2 (26a). An analysis of the deduced μ1 amino acid sequences failed to reveal any that are similar to fusogenic sequences in the fusion proteins of enveloped viruses (23); however, the myristoyl group that is attached to the amino terminus of μ1 and its amino-terminal fragments may be active in this property of membrane interaction.

The presence of the myristoylated fragments μ1N and μ1δ in ISVPs and their absence from cores suggest a model in which myristoylated polypeptides are released from ISVPs and interact with cell membranes at a specific early step in reovirus infection. A conformational change within the reovirus outer capsid allowing the release of the myristoylated fragments would seem to be an essential step in this process. The work of Borsa et al. (2, 4) indicates that some component(s) of ISVPs is capable of undergoing such a change in conformation. Our model for the penetration of reoviruses into cells is similar to that proposed for polioviruses, in which myristoylated VP4 is released from particles during virus uncoating (11) and may aid in the transfer of the viral genome across cell membranes (8, 28, 33). In the reovirus model, however, an interaction of the myristoylated fragments with cell membranes might be expected to permit entry into the cytoplasm of not just the viral genome, but the entire transcriptionally competent core particle. Future work in our laboratory will address additional features of μ1 structure and the function of μ1 and its proteolytic fragments in reovirus uncoating and entry into cells.

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