JOURNAL OF VIROLOGY, June 2006, p. 5233–5240 Vol. 80, No. 11
0022-538X/06/$08.00+0 doi:10.1128/JVI.00049-06
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

The Cytoplasmic Tail of the Influenza A Virus M2 Protein Plays a Role in Viral Assembly

Kiyoko Iwatsuki-Horimoto,[1,2] Taisuke Horimoto,[1,2] Takeshi Noda,[2,3] Maki Kiso,[1,2] Junko Maeda,[4] Shinji Watanabe,[5] Yukiko Muramoto,[1,2,6] Ken Fujii,[1,2] and Yoshihiro Kawaoka[1,2,3,5]*

Division of Virology, Department of Microbiology and Immunology,[1] and International Research Center for Infectious Diseases,[3] Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Saitama 332-0012,[2] and Department of Prion Diseases,[4] and Laboratory of Microbiology, Department of Disease Control,[6] Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan, and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706[5]

Received 7 January 2006/Accepted 8 March 2006

The viral replication cycle concludes with the assembly of viral components to form progeny virions. For influenza A viruses, the matrix M1 protein and two membrane integral glycoproteins, hemagglutinin and neuraminidase, function cooperatively in this process. Here, we asked whether another membrane protein, the M2 protein, plays a role in virus assembly. The M2 protein, comprising 97 amino acids, possesses the longest cytoplasmic tail (54 residues) of the three transmembrane proteins of influenza A viruses. We therefore generated a series of deletion mutants of the M2 cytoplasmic tail by reverse genetics. We found that mutants in which more than 22 amino acids were deleted from the carboxyl terminus of the M2 tail were viable but grew less efficiently than did the wild-type virus. An analysis of the virions suggested that viruses with M2 tail deletions of more than 22 carboxy-terminal residues apparently contained less viral ribonucleoprotein complex than did the wild-type virus. These M2 tail mutants also differ from the wild-type virus in their morphology: while the wild-type virus is spherical, some of the mutants were filamentous. Alanine-scanning experiments further indicated that amino acids at positions 74 to 79 of the M2 tail play a role in virion morphogenesis and affect viral infectivity. We conclude that the M2 cytoplasmic domain of influenza A viruses plays an important role in viral assembly and morphogenesis.

Influenza A viruses assemble and bud at the plasma membrane. However, the molecular mechanism for this process is not yet fully understood. The viral envelope contains two integral membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and one unglycosylated membrane protein, M2, as a minor component (for a review, see reference 14). The matrix protein M1 is thought to form a shell-like structure beneath the envelope. The influenza virus genome, which is single-stranded, negative-sense RNA, is fragmented into eight segments, each of which encodes one or two proteins. These viral RNA segments associate with nucleoprotein (NP) and three RNA-dependent RNA polymerase subunits (PA, PB1, and PB2), which together form viral ribonucleoprotein complexes (vRNPs). For the virus to be infectious, the vRNPs must be incorporated into budding virions, presumably by interacting with components of the virion shell.

Previous studies have indicated that the cytoplasmic tails of the HA and NA proteins affect virus morphology (12, 16) and that deletion of the cytoplasmic domains of both of these glycoproteins negatively affects the incorporation of vRNPs into virions (33). These data suggest that specific interactions occur between vRNPs, M1, and the cytoplasmic domains of these glycoproteins during virus assembly.

The M2 protein is a type III membrane protein. It forms a homotetramer and functions as a proton channel (23) that is required for efficient virus growth (27, 29). It contains three structural domains: an amino-terminal extracellular domain (comprising 24 residues), a transmembrane domain (19 residues), and a cytoplasmic domain (54 residues). The transmembrane domain is essential to the ion channel activity of M2, whereas the cytoplasmic domain is indirectly involved in this activity by stabilizing the structural pore of the protein (28). The M2 cytoplasmic domain, the longest such domain of the transmembrane proteins of influenza A viruses, is less understood. Its deletion is known to negatively affect viral replication, as indicated by the failure of viruses that lack this domain to propagate (7). In addition, amino acid substitutions found in the M2 cytoplasmic tail or in the M1 protein of mutants selected by a monoclonal antibody against the M2 ectodomain suggest a possible interaction between the cytoplasmic domain of M2 and the M1 protein (32).

Recently, McCown and Pekosz (15) showed that the M2 cytoplasmic tail likely plays a role in infectious-virus production by facilitating the efficient packaging of genome segments into influenza virions. Similarly, Imai et al. (11) reported that influenza B virus BM2, the counterpart of type A virus M2 (17), is crucial for vRNP incorporation into virions during virus assembly and may function to capture the M1-vRNP complex at the budding site. However, the specific residues in the M2 tail critical for viral infectivity and virion morphogenesis remain unknown. We therefore used reverse genetics to generate a series of mutants with incremental deletions of the M2 tail from the carboxy (C) terminus or with alanine substitutions in

* Corresponding author. Mailing address: 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5310. Fax: 81-3-5449-5408. E-mail: kawaoka@ims.u-tokyo.ac.jp.

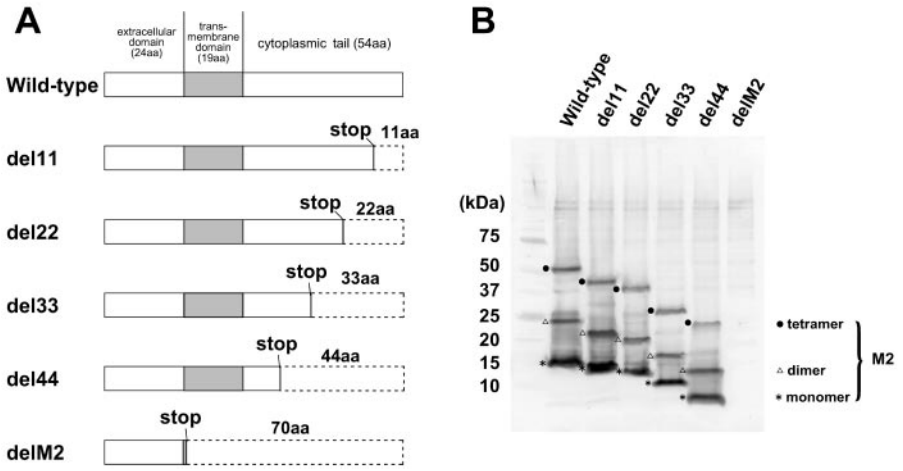

FIG. 1. Construction of M2 cytoplasmic-tail deletion mutants. (A) Schematic representation of the M2 mutants. Each mutant (del11, del22, del33, or del44) contains an 11-, 22-, 33-, or 44-amino-acid (aa) deletion from the C terminus, respectively. The mutant delM2 was constructed with deletion of 70 C-terminal residues, including the entire transmembrane and cytoplasmic regions. (B) Expression of M2 mutants in cells. Each pPolI plasmid for the expression of these M2 deletion mutants was transfected into 293T cells with four plasmids expressing PA, PB1, PB2, and NP. The M2 mutants expressed in cells were separated by electrophoresis under nonreducing conditions and detected by Western blot analysis with an anti-M2 monoclonal antibody. For mutants del11, del22, del33, and del44, monomer, dimer, and tetramer forms were observed; however, no bands were detected with the delM2 construct.

this region to assess the impacts of such mutations on virus infectivity and morphology.

MATERIALS AND METHODS

Cells. 293T human embryonic kidney cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and in minimal essential medium (MEM) containing 5% newborn calf serum, respectively. The 293T cell line is a derivative of the 293 cell line into which the gene for the simian virus 40 T antigen was inserted (8). All cells were maintained at 37°C in 5% CO_2.

Hygromycin-resistant MDCK cells stably expressing M2 protein from A/Puerto Rico/8/34 (H1N1) were established by cotransfection with plasmid pRHyg, containing the hygromycin resistance gene (10), and plasmid pCAGGS/M2, expressing the full-length M2 protein (19, 20), at a ratio of 1:1. The stable MDCK cell clone (M2CK) expressing M2 was selected in medium containing 0.15 mg/ml of hygromycin (Roche, Mannheim, Germany) by screening with indirect immunostaining using an anti-M2 (14C2) monoclonal antibody (31). The M2CK cells were cultured in MEM supplemented with 10% fetal calf serum and 0.15 mg/ml of hygromycin. In M2CK cells, the expression levels and localization of M2 were similar to those in virus-infected cells (data not shown).

Construction of plasmids. The cDNA of A/WSN/33 (WSN; H1N1) virus was synthesized by reverse transcription of viral RNA (vRNA) with an oligonucleotide complementary to the conserved 3′ end of the vRNA. The cDNA was amplified by PCR with a pair of M gene-specific oligonucleotide primers containing BsmBI sites and by using *Pfu* polymerase (Promega, Madison, WI). The resulting PCR products were cloned into the pT7Blueblunt vector (Novagen, Madison, WI). After digestion with BsmBI, the fragment containing the M gene was cloned into the BsmBI sites of the pHH21 vector, which contains the human RNA polymerase I promoter and the mouse RNA polymerase I terminator separated by BsmBI sites (19), resulting in the generation of pPolI-M (all plasmids derived from pHH21 for the expression of vRNA are referred to as PolI constructs in this report).

A series of M2 tail mutants were constructed as follows. pPol-M was first amplified by inverse PCR (22) using the back-to-back primers M972stopF (5′-CACACACGTCTCATGTTTGAGTGAGTCATTTTGTCAACATAGAGCTGGAGTAAAAAACTACC-3′) and M972stopR (5′-CACACACGTCTCCAACATCCACAGCATTCTGCTGTTCCTTTCGATATTCTTCC-3′), M939stopF (5′-CACACACGTCTCAAGAATGAGTGAAGGAACAGCAGAATGCTGTGGATGTTGACG-3′) and M939stopR (5′-CACACACGTCTCATTCTTCCCTCATAGACTCTGGCACTCCTTCCG-3′), M906stopF (5′-CACACACGTCTCCTTCTTGAGTGAGAGTGCCAGAGTCTATGAGGGAAGAATATCG-3′) and M906stopR (5′-CACACACGTCTCTAGAAGGCCCTCTTTTCAAACCGTATTTAAAGCGACG-3′), and M873stopF (5′-CACACACGTCTCATCGTTGAGTGAAATACGGTTTGAAAAGAGGGCCTTCTACG-3′) and M873stopR (5′-CACACACGTCTCGACGATAAATGCATTTGAAAAAAAGACGATCAAGAATCC-3′). These primers have a BsmBI site at their 5′ ends (underlined). The PCR products were digested with BsmBI, self-ligated, and then used to transform *Escherichia coli* strain DH5α. To construct an M2 mutant lacking the entire transmembrane and cytoplasmic regions, we used a commercial mutagenesis kit (QuikChange XL; Stratagene, La Jolla, CA) with primers pPolIwsn MdM2-5 (5′-GATTCAAGTGATTGATGAGTCATGCA-3′) and pPolIwsn MdM2-6 (5′-TGCAATGACTCATCAATCACTTGAATC-3′). The resulting constructs were designated pPolI-M2del11, pPolI-M2del22, pPolI-M2del33, pPolI-M2del44, and pPolI-delM2, each of which contained a stop codon at nucleotide positions 972 to 974, 939 to 941, 906 to 908, 873 to 875, and 786 to 788 of the M segment, which result in the deletion of 11, 22, 33, 44, and 70 residues from the C terminus of the M2 protein, respectively (Fig. 1A). A series of pPolI plasmids expressing vRNAs for M2 tail mutants containing three consecutive alanine substitutions was generated for an alanine-scanning analysis. Each construct was made by using an inverse-PCR protocol with a pair of primers containing alanine codons instead of wild-type codons at positions 74 to 76, 77 to 79, 80 to 82, 83 to 85, 86 to 88, 89 to 91, 92 to 94, or 95 to 97 (see Fig. 6). The primer sequences are available upon request. All of the constructs were sequenced to ensure that unwanted mutations were not present.

Plasmid-based reverse genetics. In all experiments, WSN genes were used to generate the viruses, using plasmids expressing eight viral RNA segments and all nine viral proteins as described by Neumann et al. (19). Transfectant viruses were harvested 48 h posttransfection, and stock viruses were made with M2CK cells.

Western blotting. Cells were lysed in Tricine sample buffer (Invitrogen, Carlsbad, CA), and proteins were resolved on 10% to 20% Tricine gels (Invitrogen) before being transferred to Immobilon-P transfer membranes (Millipore Corporation, MA). The membranes were blocked in a commercial reagent (Block-Ace; Dainippon Phamaceutical, Osaka, Japan) and then incubated with appropriately diluted primary antibodies for 2 h at room temperature or at 4°C overnight. The immunodetection kit (ABC kit; Vector Laboratories, Burlingame, CA) and Immunostain HRP-1000 (Konica Minolta Medical and Graphic, Tokyo, Japan) were used to detect antibody-antigen complexes.

Replicative properties of transfectant viruses. MDCK cells in triplicate wells of 24-well plates were incubated with virus at a multiplicity of infection (MOI) of 0.01 PFU/ml for 1 h and then overlaid with MEM containing 0.3% bovine serum albumin and 0.5 μg/ml of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin, and incubated at 37°C. At various time points, the supernatants were measured for infectious virus by plaque assays with M2CK cells in the presence of 0.5 μg/ml of TPCK-trypsin.

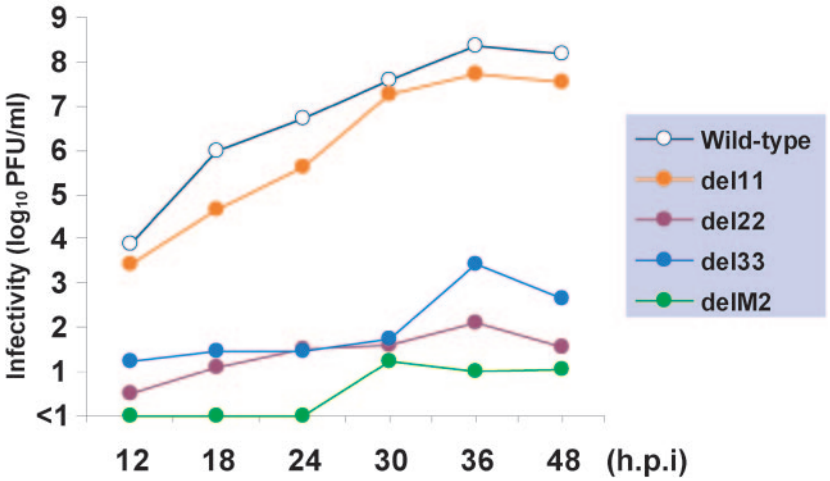

FIG. 2. Growth kinetics of M2 tail deletion mutant viruses in MDCK cells. Mutant M2 viruses were generated by reverse genetics, amplified, and titrated in M2CK cells expressing intact M2. These viruses were used to inoculate MDCK cells at an MOI of 0.01, and their growth was monitored for 48 h. Two independent experiments were performed, and representative data are shown.

Virus purification. MDCK cells were infected with virus at an MOI of 5 PFU/ml and incubated in MEM with TPCK-trypsin at 37°C. At 24 h postinfection (p.i.), the culture media were collected and clarified by centrifugation at 3,000 × *g* for 10 min. The supernatants were then transferred onto 25% sucrose in STE buffer (50 mM Tris-HCl [pH 7.2], 100 mM NaCl, and 1 mM EDTA) and ultracentrifuged in a Beckman SW40 rotor (270,000 × *g*) for 2 h at 4°C. The virus pellets were resuspended in a small aliquot of STE buffer, lysed in the sample buffer, and subjected to electrophoresis through a 10% to 20% Tricine gel.

Electron microscopy. For thin-section electron microscopy, MDCK cells were infected with virus at an MOI of 5 PFU/ml and incubated in MEM with TPCK-trypsin at 37°C. Approximately 12 h p.i., the MDCK cells were washed with phosphate-buffered saline, prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C, and then fixed with 2% osmium tetroxide in the same buffer for 1 h at 4°C. Specimens were subsequently stained en bloc with 1% aqueous uranyl acetate for 30 min at 4°C and processed as previously described (21).

For negative staining, the culture medium from MDCK cells infected with wild-type or mutant viruses was collected at 24 h p.i. onto a Formvar-coated copper grid, stained with 2% phosphotungstic acid solution, and examined with a JEM-1200EX electron microscope at 80 kV.

RESULTS

Generation of M2 cytoplasmic-tail deletion mutant viruses by reverse genetics. To understand the biologic functions of the cytoplasmic tail of the influenza A virus M2 protein, we constructed a series of PolI plasmids that encoded M2 mutants with cytoplasmic-tail deletions (Fig. 1A). Prior to the reverse-genetics experiments, we assessed the expression of each M2 mutant by transfecting 293T cells with each PolI plasmid, together with plasmids expressing PB1, PB2, PA, and NP. M2 mutant protein expression in the cell lysates was analyzed by Western blotting with an anti-M2 monoclonal antibody. As shown in Fig. 1B, M2 mutant proteins were detected as monomers, dimers, and tetramers, except with the pPolI-delM2 construct. To assess cell surface expression of these mutant proteins, we performed an immunostaining assay with the anti-M2 monoclonal antibody. The M2 mutant del11, del22, del33, and del44 proteins were detected on the cell surface without appreciable differences in their expression levels, whereas no signal was detected with the delM2 construct (data not shown). Thus, the M2 mutant proteins, with the exception of delM2, were translated and transported to the cell surface, as would occur with the wild-type M2 protein.

We next attempted to generate M2 mutant viruses by plasmid-based reverse genetics (19). Each of the PolI plasmids, pPolI-M2del11, -del22, -del33, -del44, and -delM2, was used for transfection in place of the pPolI-M plasmid that bears the wild-type M segment. Virus was readily generated with the

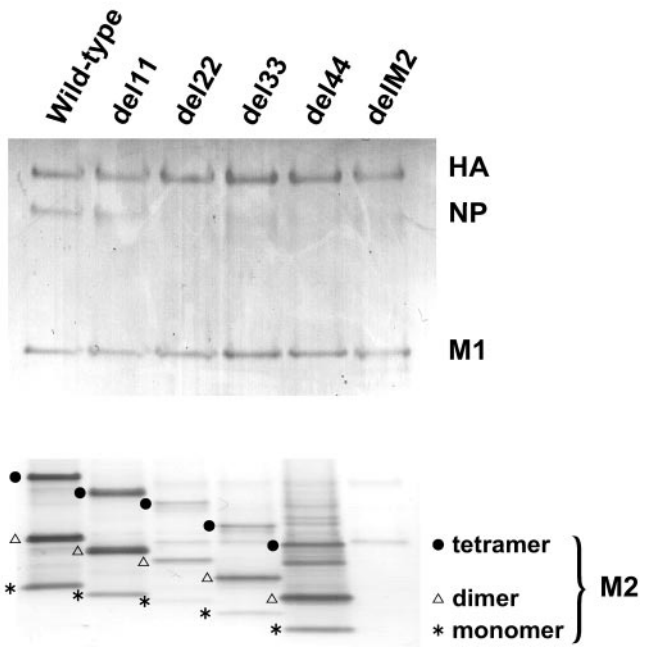

FIG. 3. Protein contents of M2 tail deletion mutant viruses. Viruses generated by reverse genetics were amplified in M2CK cells and used to infect MDCK cells at an MOI of 5. At 24 h postinfection, the viruses in the culture supernatant were purified and lysed, and the viral proteins were separated by electrophoresis under nonreducing conditions. The gel was stained with Coomassie brilliant blue to visualize the viral proteins, and M2 proteins were detected in an immunoblot assay with an anti-M2 (14C2) monoclonal antibody.

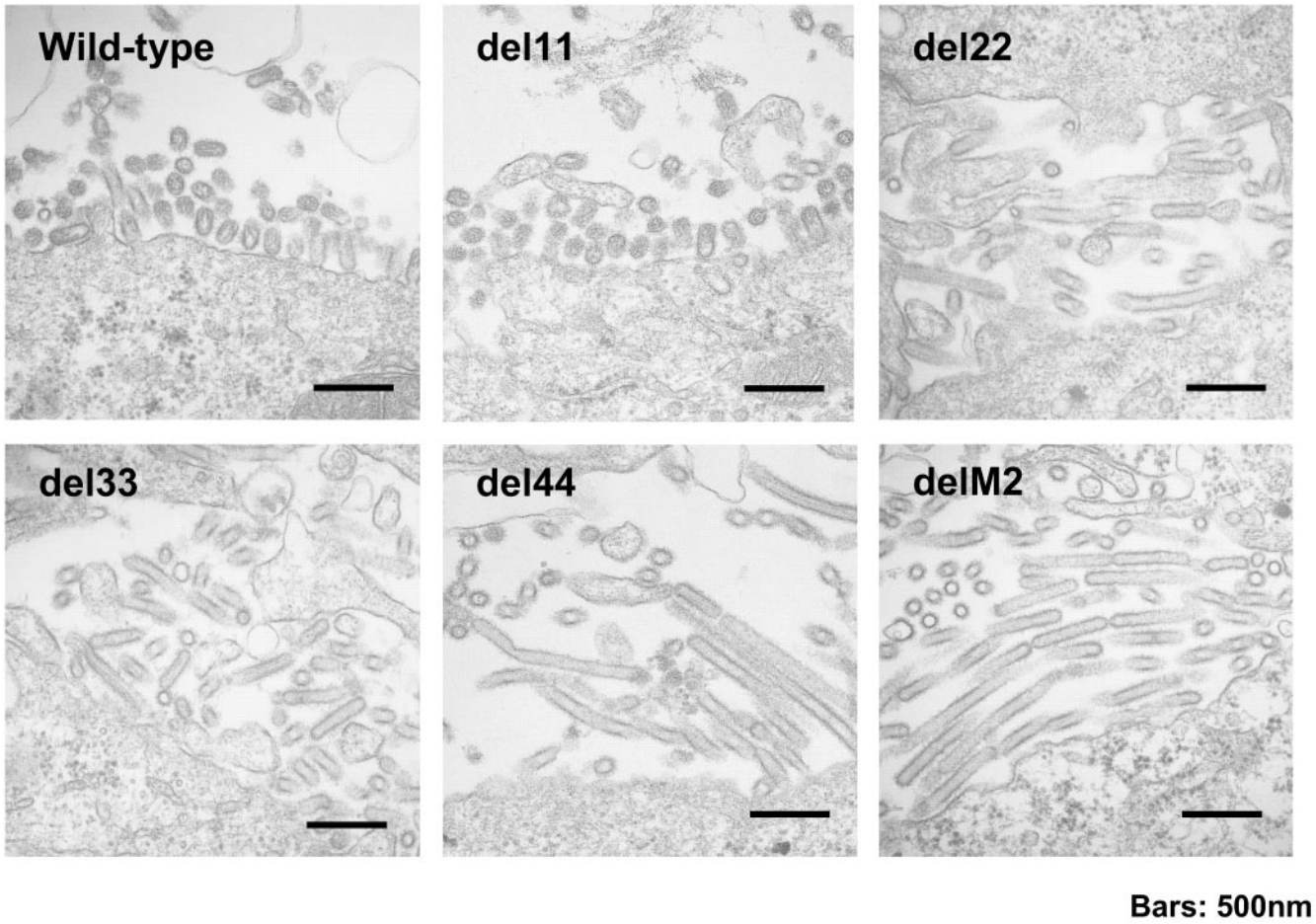

FIG. 4. Thin-section electron microscopy of MDCK cells infected with M2 tail deletion mutant viruses. The virus-infected cells were fixed 12 h postinfection and then processed for testing.

pPolI-M2del11 construct, as well as with the pPolI-M construct (positive control); however, no virus was detected with the -del22, -del33, -del44, and pPolI-delM2 constructs. These results indicate that deletion of 22 or more C-terminal residues negatively affects the function of the M2 protein.

Failure to generate a mutant virus with pPolI-delM2 is inconsistent with our previous study, in which we were able to generate a mutant virus whose M2 gene lacks the regions encoding both the transmembrane and cytoplasmic domains (29). It is possible that the efficiency of virus generation was lower in the current experiment than in the previous study, because we used an M gene in this study that was derived from the WSN strain rather than the A/Udorn/307/72 (H3N2) strain we used in the previous study. To increase the efficiency of virus generation for M2 mutant viruses, we made M2CK cells stably expressing the wild-type M2 protein. We then inoculated these M2CK cells with the supernatants of plasmid-transfected 293T cells. By 48 h postinoculation, cytopathic effects were evident in cells infected with all of the M2 mutant constructs. The virus titers in these culture fluids were comparable to that of the wild-type virus: 5.0×10^7 PFU/ml for M2del11, 8.3×10^7 for del22, 1.3×10^8 for del33, 1.1×10^8 for del44, 7.2×10^7 for delM2, and 2.2×10^8 for the wild-type virus.

Growth properties of the M2 tail deletion mutant viruses. We compared the growth properties of the M2 tail mutant viruses with those of wild-type virus in MDCK cells (Fig. 2). Viruses produced in M2CK cells were used to infect MDCK cells at an MOI of 0.01, and their growth kinetics were monitored. The M2del11 virus grew nearly as well as the wild-type virus, whereas the mutant viruses M2dell22, M2del33, and delM2 grew significantly less well ($P < 0.05$ versus wild-type virus). Two independent experiments with the M2del44 mutant virus resulted in the emergence of revertants, which grew efficiently due to a substitution that resulted in the alteration of the introduced stop codon (data not shown). These results confirm our previous finding (7) that the cytoplasmic tail of the M2 protein plays an important role in viral replication.

Protein contents of the M2 tail deletion mutant viruses. To gain insight into the molecular basis for the poor growth of the M2 tail mutant viruses that had more than 22 amino acid residues deleted from their C termini, we examined the protein content of each mutant. Mutant and wild-type viruses that had been propagated in M2CK cells were used to infect MDCK cells at an MOI of 5, purified from the culture supernatant, and subjected to electrophoresis to separate the viral proteins (Fig. 3, upper panel). Appreciable differences in the amounts of NP

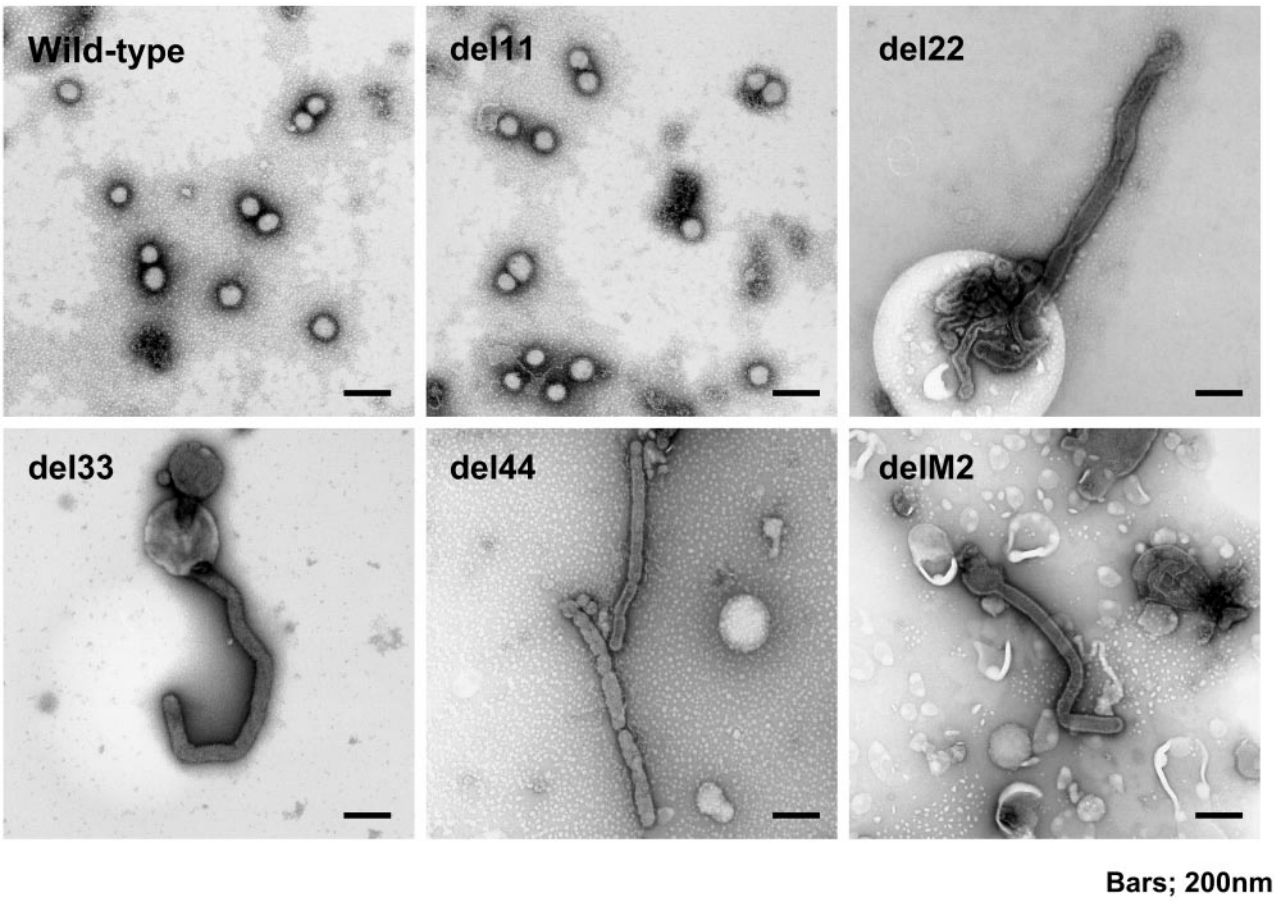

FIG. 5. Electron microscopy of the M2 tail deletion mutant virions. MDCK culture supernatants containing M2 mutant or wild-type viruses were observed after negative staining.

among the test viruses were detected, with higher NP levels in the wild-type and M2del11 viruses but substantially lower levels in the others. Equivalent amounts of HA and M1 proteins were nonetheless present in these viruses (Fig. 3, upper panel). Western blot analyses showed that M2 proteins were present in virions of all test viruses, with the exception of the delM2 mutant. There appeared to be less M2 protein present with the M2del22 and del33 mutants than with the others (Fig. 3, lower panel). The additional bands derived from the del44 virus likely reflected the presence of a revertant in which a mutation had abolished the introduced stop codon, as described above. These results suggest two possibilities: (i) deletions of more than 22 C-terminal residues from the M2 cytoplasmic tail affected the incorporation efficiency of vRNPs into virions, as has been suggested by McCown and Pekosz (15), or (ii) these deletions affected virion morphology, resulting in a concomitant reduction of NP/HA ratios, NP/M ratios, or both.

Morphology of the M2 tail deletion mutant viruses. To examine the effect of the M2 tail on virion morphogenesis, we observed virus-infected cells by thin-section electron microscopy (Fig. 4). In cells infected with either the wild-type or M2del11 virus, we observed spherical virions budding at the cell surface. Although all of the wild-type virions were filled with electron-dense materials, some of the M2del11 virions were empty. By contrast, in cells infected with M2del22, -del33, -del44, or delM2 virus, we observed filamentous particles that lacked electron-dense materials. Negative staining of both the M2del11 and wild-type viruses showed them to indeed be spherical, with diameters between 100 and 120 nm (Fig. 5). Similar staining of M2del22, -del33, -del44, and delM2 showed them to be filamentous, approximately 500 to 1,500 nm in length, and 100 nm in width (Fig. 5). These results indicate that the M2 cytoplasmic tail is involved in virion morphogenesis.

Generation and morphological properties of M2 tail mutant viruses possessing alanine substitutions. The analysis of M2 tail deletion mutants suggested that the M2 amino acids at positions 76 to 86 may be involved in virion morphogenesis. We therefore performed an alanine scanning at each of the three consecutive alanine residues within this 24 residue C-terminal region (Fig. 6). Two of the eight mutants generated by reverse genetics (mut 1 and 2) grew less efficiently than the other mutants (Fig. 7), whereas all of the others replicated as efficiently as the wild-type virus, indicating that the amino acids at positions 74 to 79 of the M2 tail are important for optimal virus growth.

We then assessed virion morphology among these M2 tail/alanine substitution mutants by electron microscopy. Interestingly, the virions of mut 1 and 2 appeared fragile and irregular (Fig. 8), with diameters that were significantly smaller than that of the wild-type virus (Fig. 9). A small population of mut

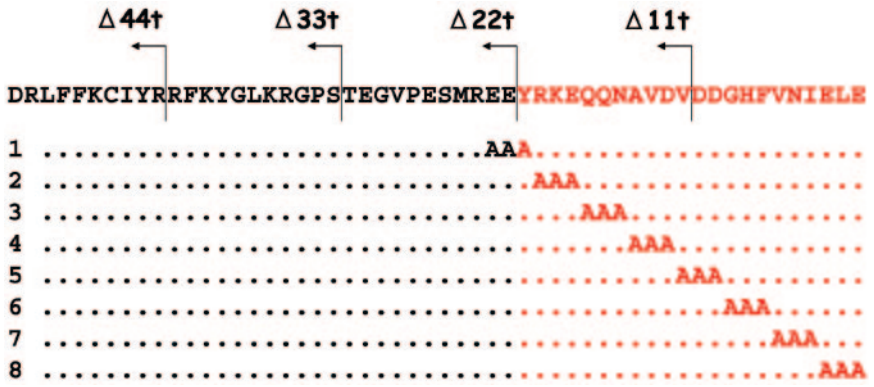

FIG. 6. Construction of M2 tail mutants possessing alanine substitutions. Wild-type amino acid sequences of the M2 tail are shown in the upper row. Each mutant (1 to 8) contained three consecutive alanine substitutions at the indicated positions. The 22 amino acids at the C terminus that were deleted in the M2-del22 mutant are highlighted in red.

3 also formed virions with similar morphology. However, filamentous particles were not observed in any of these mutant viruses. By contrast, normal spherical particles were evident with mut 4, 5, 6, 7, and 8 and the wild-type virus. These results indicate that the amino acids at positions 74 to 79 of the M2 cytoplasmic tail are involved in virion morphogenesis, affecting viral infectivity.

DISCUSSION

Influenza A virus assembly and budding is an area of active research. Two glycoproteins, HA and NA, are known to accumulate at the apical plasma membrane with their apical sorting signals (13) and to associate with lipid raft microdomains, enriched in sphingomyelin and cholesterol. Such findings have led to the suggestion that lipid rafts may serve as a platform for virus budding (2, 26). In keeping with these observations is the recent report that NP is also targeted to the apical membrane and the lipid rafts (6). On the other hand, even though M2 has a cholesterol-binding domain, the majority of this protein does not associate with lipid rafts (25). However, our previous reverse-genetics study indicated that despite low concentrations of M2 in virions, the cytoplasmic tail of this protein plays a critical role in viral replication (7). Here, we demonstrate that the M2 cytoplasmic domain also plays a role in virus morphogenesis and confirm a previous report that alteration of this domain results in reduction of vRNP content in virions. Our findings are further supported by previous data demonstrating a requirement for the cytoplasmic tails of HA and NA for normal vRNP packaging and virion morphology (12, 16, 33). Thus, the cytoplasmic tails of all three membrane proteins cooperatively play roles in proper virus assembly.

At the final stage of viral replication, the envelope proteins (HA, NA, and M2), M1, and vRNPs (and NS2) must be brought together to the budding site, presumably by lipid rafts. Previous studies have indicated that M1, the most abundant viral protein in virions, interacts with vRNPs (1, 3, 30) and the cytoplasmic and transmembrane domains of HA and NA (9, 24) to create a bridge between the viral envelope and the vRNPs, which results in the formation of particles possessing all of the viral components necessary for infectivity (for a review, see reference 18). The present study showed an appar-

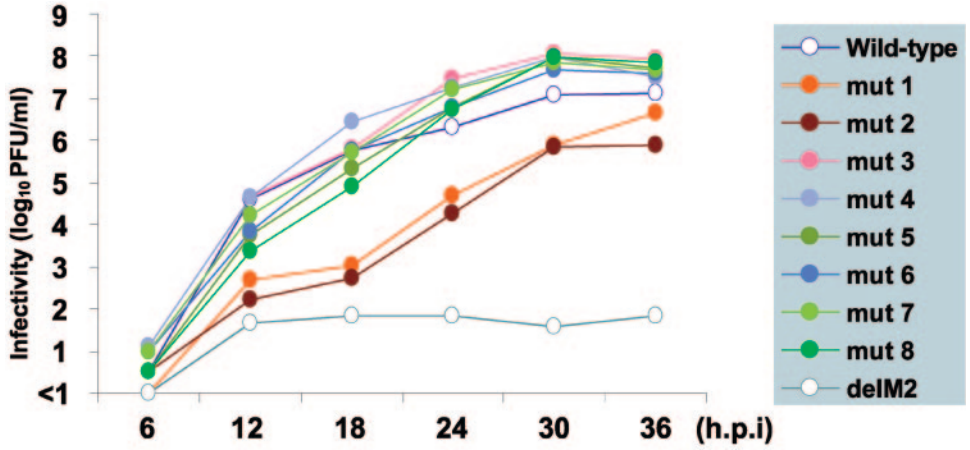

FIG. 7. Growth kinetics in MDCK cells of M2 tail mutant viruses possessing alanine substitutions. Mutant viruses were generated by reverse genetics with 293T cells, amplified in M2CK cells, and used to inoculate MDCK cells at an MOI of 0.01. Growth was monitored for 36 h. Two independent experiments were performed, and representative data are shown.

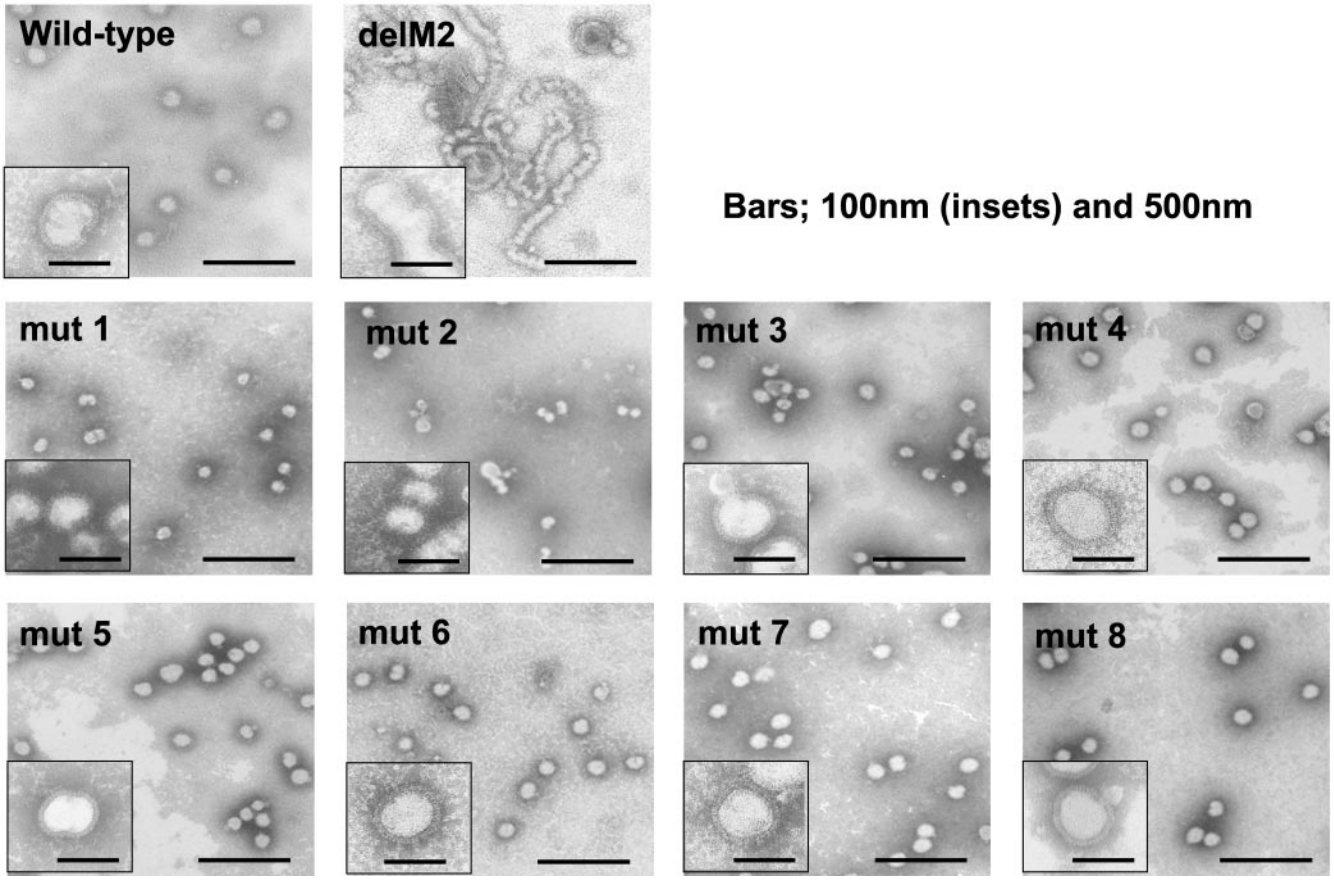

FIG. 8. Electron microscopy of the M2 tail mutant virions possessing alanine substitutions. Cell culture supernatants containing M2 mutant or wild-type viruses were observed after negative staining. Representative particles of each mutant or wild-type virus are also shown at a higher magnification.

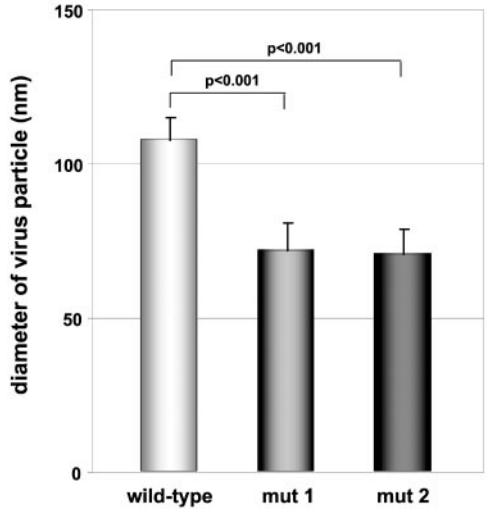

FIG. 9. Comparison of virion size by electron microscopy. Diameters of mutants 1 and 2 are shown and compared to that of wild-type virus ($n = 25$). The mutants are statistically smaller than the wild type ($P < 0.001$; Student t test).

ent reduction in vRNP content in mutant viruses that carried a large deletion in the M2 cytoplasmic domain. It is possible that the M2 cytoplasmic domain directly interacts with M1, increasing the stability of the M1 shell-like structure, which is thought to be required for efficient vRNP incorporation (32). Alternatively, the M2 cytoplasmic tail may "grab" the vRNPs, directly interacting with them via its long cytoplasmic tail, leading to vRNP incorporation into virions. Although there is no evidence for such interaction, M2 has been copurified with nucleocapsid, likely due to an association between M2 and the nucleocapsid components (5).

It is possible that the drastic reduction in infectivity of mutant viruses with large M2 cytoplasmic deletions stems from a defect independent of vRNP packaging. For example, the M2 tail deletions may negatively affect ion channel activities, which could reduce virus infectivity. Indeed, a previous study has revealed that 16 and 36 C-terminal residue deletions of the M2 tail reduce the ion channel activity of this protein without affecting its expression levels on the cell surface, whereas 6-, 21- and 26-residue deletions have no such effect, indicating that ion channel activity can be altered by some cytoplasmic mutations and deletions (28). Thus, the decreased infectivity of our M2 tail mutants may result from reduced ion channel activity in addition to a defect in vRNP incorporation.

Our assessment of virus morphogenesis in this study re-

vealed that M2 mutants with more than 22 C-terminal residue deletions exhibit a more filamentous morphology than the wild-type virus. This finding is contrary to that of McCown and Pekosz (15), who described the particles of their M2 mutant with a 28-residue tail deletion as spherical and similar to wild-type virus in morphology. The reason for this discrepancy is unknown. In our study, morphological changes were also observed with two M2 tail mutants that had undergone alanine substitutions, although these changes were less drastic. Almost all of the particles of these mutant viruses appeared to have lost integrity and seemed fragile. Therefore, we conclude that the M2 tail domain amino acids at positions 74 to 79 play an important role in virus morphogenesis. This finding is somewhat analogous to that of a previous study, in which a mutant virus lacking both its HA and NA cytoplasmic tails exhibited an altered morphology (12). As described above, since the M2 cytoplasmic domain likely interacts with M1 and M1 is known to affect virion morphology (4), it is possible that alteration of the M2 domain affects the structure of the M1 shell and, thus, the virion shape. Alternatively, given Schroeder's recently proposed hypothetical mechanism in which M2 is predicted to control pinching off of virus budding from cells (25), it may be that M2 tail deletions or amino acid substitutions alter this pinching off activity, leading to morphological changes in the virions. Clearly, the data to date indicate that the cytoplasmic tails of all three integral membrane proteins participate in virion morphogenesis.

In conclusion, M2 is a multifunctional protein whose transmembrane domain plays a critical role in ion channel activity and whose cytoplasmic domain is required for vRNP incorporation and virion morphogenesis.

ACKNOWLEDGMENTS

We thank Sue Watson for editing the manuscript. We also thank Robert A. Lamb for providing us with the anti-M2 (14C2) monoclonal antibody.

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, by CREST (Japan Science and Technology Agency), and by Public Health Service research grants from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Ali, A., R. T. Avalos, E. Ponimaskin, and D. P. Nayak.** 2000. Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. J. Virol. **74:**8709–8719.
2. **Barman, S., L. Adhikary, A. K. Chakrabarti, C. Bernas, Y. Kawaoka, and D. P. Nayak.** 2004. Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza A virus neuraminidase in raft association and virus budding. J. Virol. **78:**5258–5269.
3. **Baudin, F., I. Petit, W. Weissenhorn, and R. W. Ruigrok.** 2001. In vitro dissection of the membrane and RNP binding activities of influenza virus M1 protein. Virology **281:**102–108.
4. **Bourmakina, S. V., and A. Garcia-Sastre.** 2003. Reverse genetics studies on the filamentous morphology of influenza A virus. J. Gen. Virol. **84:**517–527.
5. **Bron, R., A. P. Kendal, H.-D. Klenk, and J. Wilschut.** 1993. Role of the M2 protein in influenza virus membrane fusion: effects of amantadine and monensin on fusion kinetics. Virology **195:**808–811.
6. **Carrasco, M., M. J. Amorim, and P. Digard.** 2004. Lipid raft-dependent targeting of the influenza A virus nucleoprotein to the apical plasma membrane. Traffic **5:**979–992.
7. **Castrucci, M. R., and Y. Kawaoka.** 1995. Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein. J. Virol. **69:**2725–2728.
8. **Dubridge, R. B., P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller, and M. P. Calos.** 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7:**379–387.
9. **Enami, M., and K. Enami.** 1996. Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein. J. Virol. **70:**6653–6657.
10. **Hatta, M., H. Goto, and Y. Kawaoka.** 2004. Influenza B virus requires BM2 protein for replication. J. Virol. **78:**5576–5583.
11. **Imai, M., S. Watanabe, A. Ninomiya, M. Obuchi, and T. Odagiri.** 2004. Influenza B virus BM2 protein is a crucial component for incorporation of viral ribonucleoprotein complex into virions during virus assembly. J. Virol. **78:**11007–11015.
12. **Jin, H., G. P. Leser, J. Zhang, and R. A. Lamb.** 1997. Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. EMBO J. **16:**1236–1247.
13. **Kundu, A., R. T. Avalos, C. M. Sanderson, and D. P. Nayak.** 1996. Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells. J. Virol. **70:**6508–6515.
14. **Lamb, R. A., and R. M. Krug.** 2001. Orthomyxoviridae: the viruses and their replication, p. 1487–1531. *In* D. M. Knipe, et al. (ed.), Fields virology, 4th ed. Lippincott-Raven, Philadelphia, Pa.
15. **McCown, M. F., and A. Pekosz.** 2005. The influenza A virus M2 cytoplasmic tail is required for infectious virus production and efficient genome packaging. J. Virol. **79:**3595–3605.
16. **Mitnaul, L. J., M. R. Castrucci, K. G. Murti, and Y. Kawaoka.** 1996. The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. J. Virol. **70:**873–879.
17. **Mould, J. A., R. G. Paterson, M. Takeda, Y. Ohigashi, P. Venkataraman, R. A. Lamb, and L. H. Pinto.** 2003. Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. Dev. Cell **5:**175–184.
18. **Nayak, D. P., E. K.-W. Hui, and S. Barman.** 2004. Assembly and budding of influenza virus. Virus Res. **106:**147–165.
19. **Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. USA **96:**9345–9350.
20. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199.
21. **Noda, T., H. Sagara, E. Suzuki, A. Takada, H. Kida, and Y. Kawaoka.** 2002. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. J. Virol. **76:**4855–4865.
22. **Ochman, H., A. S. Gerber, and D. L. Hartl.** 1988. Genetics applications of an inverse polymerase chain reaction. Genetics **120:**621–623.
23. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** 1992. Influenza virus M2 protein has ion channel activity. Cell **69:**517–528.
24. **Ruigrok, R. W., A. Barge, P. Durrer, J. Brunner, K. Ma, and G. R. Whittaker.** 2000. Membrane interaction of influenza virus M1 protein. Virology **267:**289–298.
25. **Schroeder, C., H. Heider, E. Moncke-Buchner, and T.-I. Lin.** 2005. The influenza virus ion channel and maturation cofactor M2 is a cholesterol-binding protein. Eur. Biophys. J. **34:**52–66.
26. **Takeda, M., G. P. Leser, C. J. Russell, and R. A. Lamb.** 2003. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. Proc. Natl. Acad. Sci. USA **100:**14610–14617.
27. **Takeda, M., A. Pekosz, K. Shuck, L. H. Pinto, and R. A. Lamb.** 2002. Influenza A virus M2 ion channel activity is essential for efficient replication in tissue culture. J. Virol. **76:**1391–1399.
28. **Tobler, K., M. L. Kell, L. H. Pinto, and R. A. Lamb.** 1999. Effect of cytoplasmic tail truncations on the activity of the M2 ion channel of influenza A virus. J. Virol. **73:**9695–9701.
29. **Watanabe, T., S. Watanabe, H. Ito, H. Kida, and Y. Kawaoka.** 2001. Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity. J. Virol. **75:**5656–5662.
30. **Ye, Z., T. Liu, D. P. Offringa, J. McInnis, and R. A. Levandowski.** 1999. Association of influenza virus matrix protein with ribonucleoproteins. J. Virol. **73:**7467–7473.
31. **Zebedee, S. L., and R. A. Lamb.** 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J. Virol. **62:**2762–2772.
32. **Zebedee, S. L., and R. A. Lamb.** 1989. Growth restriction of influenza A virus by M2 protein antibody is genetically linked to the M1 protein. Proc. Natl. Acad. Sci. USA **86:**1061–1065.
33. **Zhang, J., G. P. Leser, A. Pekosz, and R. A. Lamb.** 2000. The cytoplasmic tails of the influenza virus spike glycoproteins are required for normal genome packaging. Virology **269:**325–334.