

Cytoplasmic Domain of Influenza B Virus BM2 Protein Plays Critical Roles in Production of Infectious Virus[▽]

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Influenza B virus BM2 is a type III integral membrane protein that displays H⁺ ion channel activity. Analysis of BM2 knockout mutants has suggested that this protein is a necessary component for the capture of M1-viral ribonucleoprotein (vRNP) complex at the plasma membrane and for incorporation of vRNP complex into the virion during the assembly process. BM2 comprises 109 amino acid residues and possesses a longer cytoplasmic domain than the other 3 integral membrane proteins (hemagglutinin, neuraminidase, and NB). To explore whether the cytoplasmic domain of BM2 is important for infectious virus production, a series of BM2 deletion mutants lacking three to nine amino acid residues at the carboxyl terminus, BM2Δ107-109, BM2Δ104-109, and BM2Δ101-109, was generated by reverse genetics. Intracellular transport and incorporation into virions were indistinguishable between truncated BM2 proteins and wild-type BM2. The BM2Δ107-109 mutant produced levels of infectious virus similar to those of wild-type virus and displayed a spherical shape. However, the BM2Δ104-109 and BM2Δ101-109 mutants produced viruses containing dramatically reduced vRNP complex, as with BM2 knockout mutants, and formed enlarged, irregularly shaped virions. Moreover, gradient separation of membranes indicated that membrane association of M1 from mutants was greatly affected by carboxyl-terminal truncations of BM2. Studies of alanine substitution mutants further suggested that amino acid sequences in the 98–109 region are variable while those in the 86–97 region are a prerequisite for innate BM2 function. These results indicate that the cytoplasmic domain of the BM2 protein is required for firm association of the M1 protein with lipid membranes, vRNP complex incorporation into virions, and virion morphology.

Influenza A and B viruses are enveloped viruses that assemble at the plasma membrane of infected cells and release by budding. The core consists of a helical ribonucleocapsid (RNP) that contains the eight RNA segments encapsidated with nucleoprotein (NP) and polymerase proteins. The envelope accommodates three to four different transmembrane proteins: hemagglutinin (HA) glycoprotein; neuraminidase (NA) glycoprotein; and either M2 in influenza A virus or BM2 in influenza B virus. Influenza B virus contains an additional transmembrane protein, NB. The M1 matrix protein forms a layer underneath the viral envelope and also surrounds the viral RNP (vRNP) complex. M1 is thought to play a pivotal role in virus assembly, interacting with the vRNP complex and the cytoplasmic domain (endodomain/tail) of glycoproteins, as well as with membranes of infected cells (30).

The cytoplasmic domains of the HA and NA glycoproteins have also been shown to play regulatory roles in virus assembly. In the influenza A virus, HA and NA significantly enhance the membrane association of M1 (6, 10) and recruit M1 to lipid rafts (1, 53). Deletion of the cytoplasmic domains of the HA and NA glycoproteins has drastic effects on virus morphology (1, 17, 27) and genome packaging in virions (52). Furthermore,

a recent study demonstrated that the cytoplasmic domains of HA and NA are required for efficient incorporation of M1 into virus-like particles (4). These observations have suggested that the specificity of the assembly process is governed by interactions between cytoplasmic domains of glycoproteins and the M1 protein. The importance of the cytoplasmic domain of viral glycoproteins in virus assembly and budding has already been confirmed for other enveloped negative-strand RNA viruses (7, 26, 33, 40, 41, 46).

The BM2 protein of influenza B virus is a phosphoprotein that is synthesized in the late phase of infection and is incorporated into the virion as a relatively minor component (32). This protein, like M2 in influenza A virus, functions as a proton channel that is essential for dissociation of vRNP from M1 during the uncoating process and for preservation of the native conformation of HA during transport to the cell surface (28). Reverse-genetics studies have shown that BM2 knockout virus lacking the ability to synthesize the BM2 protein does not grow substantially in cell culture, indicating that the BM2 protein is necessary for the viral replication cycle (12, 14). In addition, BM2-knockout virus shows dramatically reduced incorporation of vRNP and M1 into virions (14). These findings further suggest that BM2 plays crucial roles in the virion assembly process in addition to the uncoating process.

BM2 is a tetrameric type III transmembrane protein with a C_{in}N_{out} orientation and comprises a 23-amino-acid transmembrane domain and an 86-amino-acid cytoplasmic domain (35, 47). The transmembrane domain of BM2 can act as an ion channel for protons (28). Mutant virus with a BM2 protein

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carrying an internal deletion of 30 amino acids in the cytoplasmic domain was found to incorporate dramatically reduced amounts of vRNP and M1 (14), implying that the cytoplasmic domain of BM2 might play an important role in viral assembly. On the other hand, for influenza A virus, truncation of the carboxy-terminal 28 amino acids from the cytoplasmic domain of the M2 protein greatly reduces virus infectivity and results in reduced incorporation of NP and viral RNA into virions (25). Similarly, truncation of the carboxy-terminal 22 amino acids from the M2 cytoplasmic domain reduces infectious virus titers and results in production of filamentous particles (16). A recent study identified the sequences of the M2 cytoplasmic domain that were required for specific incorporation of vRNP into virions and demonstrated interaction of the M2 cytoplasmic domain with the M1 protein (24).

To define in greater detail the role of the cytoplasmic domain in the assembly process, we generated a series of truncation and substitution mutants in the BM2 cytoplasmic domain using reverse genetics. The results showed that truncation of six amino acids from the carboxyl-terminal end of the BM2 protein affects the membrane association of M1 and greatly reduces incorporation of the vRNP complex into virions, resulting in altered particle morphology. We also found that amino acid substitutions within the carboxyl-terminal region result in a failure to incorporate the vRNP complex into virions. Given these findings, the cytoplasmic domain of BM2 protein may play key roles in the generation of infectious virus.

MATERIALS AND METHODS

Plasmids. The plasmid pCAGGS/BM2, which encodes the BM2 gene of B/Yamagata/1/73 (B/Yamagata) virus, has been described previously (32). To generate BM2 deletion constructs, the BM2 open reading frame was cloned into the pT7BlueBlunt vector (Novagen, Madison, WI) and the mutated BM2 genes were amplified by inverse PCR (primer sequences are available on request). PCR products were then cloned into the pCAGGS/MCS eukaryotic expression plasmid (31). The resulting constructs were designated pCBM2Δ104-109.

Cells and antibodies. MDCK cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS). Human embryonic kidney 293T cells and CK/BM2 cells (14) were cultured in Dulbecco's modified Eagle's medium containing 10% FCS. Stable cell lines expressing the wild-type or mutated BM2 protein were established as described previously (14). Briefly, MDCK cells were cotransfected with plasmid pCB7, encoding hygromycin resistance, and with the appropriate pCAGGS construct at a ratio of 3:1. Stable MDCK cell clones were selected in medium containing hygromycin at 0.2 mg/ml (Invitrogen, Carlsbad, CA) and were screened by an indirect immunofluorescence assay (IFA). Polyclonal anti-B/Yamagata virus antibodies have been described previously (32). A synthetic peptide, LSDNMERLSDHIVIEGLSAE, corresponding to amino acids 74 to 93 in the BM2 open reading frame of B/Yamagata virus, was synthesized by Bio-Synthesis (Lewisville, TX). For preparation of the antiserum specific for the influenza B virus BM2 protein, the peptide was coupled with keyhole limpet hemocyanin and mice were immunized. The antiactin antibody was obtained from Sigma (St. Louis, MO).

Reverse genetics. Plasmids to express mutant BM2 genes with truncations or substitutions were constructed as follows. Mutated M genes were amplified by PCR from pT7BlueBlunt containing the B/Yamagata M gene and were then digested using BsmBI (primer sequences provided on request). The BsmBI-digested fragment was cloned into the BsmBI sites of RNA expression polymerase I (Pol I) plasmids. All constructs were sequenced to ensure that no unwanted mutations were present. Transfectant influenza viruses were generated as described previously (14). Briefly, 293T cells (0.8×10^6 cells) were plated the day before transfection. Twelve plasmids (eight Pol I constructs for eight RNA segments and four protein expression constructs for three polymerase proteins and NP) were mixed with transfection reagent (trans IT LT-1; Panvera, Madison, WI) at 2 μ l/ μ g of DNA and then added to 293T cells in Dulbecco's modified Eagle's medium–10% FCS. At 16 h posttransfection, medium was replaced by Opti-MEM I (Invitrogen) containing 10 μ g/ml of trypsin. At 48 h posttransfec-

tion, viruses in supernatants were collected and propagated in CK/BM2 cells in Opti-MEM I containing 10 μ g/ml trypsin.

IFA. MDCK cells grown on coverslips were infected with wild-type and mutant viruses and incubated for 11 h at 34°C. Cells were washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Background staining was blocked with 10% goat serum in PBS for 1 h. Cells were then incubated with anti-BM2 antibodies. After incubation for 1 h, cells were washed with PBS and incubated for 1 h with Alexa 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Invitrogen). Coverslips were mounted on glass slides and examined using confocal microscopy (model LSM 510; Carl Zeiss, Oberkochen, Germany).

Western blotting. MDCK cells were infected with virus at a multiplicity of infection (MOI) of 10 PFU/cell and incubated at 34°C. At 10 h postinfection (p.i.), cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) and maintained for 30 min on ice. After clarification by centrifugation, the supernatant was dissolved in SDS sample buffer (50 mM Tris-HCl [pH 6.8], 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and bromophenol blue) at 95°C for 5 min, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and electroblotted to Immobilon-P paper (Millipore, Billerica, MA). After incubation with the appropriate antibody (antiserum to BM2 or B/Yamagata virion), the membrane was treated with horseradish peroxidase-conjugated anti-mouse IgG for influenza B virus BM2 or HRP-conjugated anti-rabbit IgG for B/Yamagata virion, respectively, and visualized by enhanced chemiluminescence according to protocols provided by GE Healthcare Life Sciences (Little Chalfont, England). Band intensities were measured with a LightCapture system (model AE-6961; ATTO, Tokyo, Japan) using a CS analyzer (ATTO).

Analysis of the protein composition of virions. MDCK cells grown in 10-cm dishes were infected with wild-type or mutant virus at an MOI of 5 PFU and cultured in Opti-MEM I at 34°C. At 24 h p.i., supernatants from cell culture were harvested and clarified by centrifugation at $1,620 \times g$ for 10 min at 4°C. Virions were purified by centrifugation through 30% sucrose in a SW41 Ti rotor (Beckman Coulter, Fullerton, CA) at $150,000 \times g$ for 1 h at 4°C. Pellets were then suspended in 50 mM Tris-HCl (pH 7.5), and the samples were analyzed by SDS-PAGE. Proteins were detected by using Coomassie brilliant blue and Western blotting using anti-BM2 antibody. The profiles of the stained gels were digitized and were quantified as described previously (14).

Real-time PCR. RNA was extracted from purified virions using an RNeasy kit (Qiagen, Hilden, Germany) in accordance with the instructions of the manufacturer. The cDNAs were synthesized by reverse transcription of vRNA with an oligonucleotide complementary to the conserved 3' end of vRNA. Primer and probe sequences for B/Yamagata virus HA genes have been described previously (14). Amplification and detection by real-time PCR were performed using the Chromo-4 real-time PCR detection system (Bio-Rad, Hercules, CA). PCR was performed with 50 μ l of reaction mixture consisting of 1 \times PCR Gold buffer (GeneAmp Gold PCR reagent kit; Applied Biosystems, Foster City, CA), 250 μ M of each deoxynucleoside triphosphate, 5 mM MgCl₂, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.2 μ M each of forward and reverse primers, 0.1 μ M of probe, and 5 μ l of cDNA. Reaction conditions were set at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The standard curve for this assay was calculated using a series of 10-fold dilutions of Pol I plasmids encoding the B/Yamagata virus HA gene.

Protease treatment of virions. Purified virions in 50 mM Tris-HCl (pH 7.5) were digested with 0.05 mg/ml trypsin (sequencing grade; Roche, Mannheim, Germany) at 25°C. After incubation for 2 h, digestion was stopped by adding 4-(2-aminoethyl)-benzenesulfonyl fluoride (Roche) to a 4 mM concentration. Samples were dissolved in SDS sample buffer and subjected to SDS-PAGE.

Electron microscopy. A droplet of purified virions was placed on a 400-mesh copper grid (Veco, Eiersbeck, The Netherlands) coated with collodion and then negatively stained with 1% uranylacetate. Images were obtained using an H-7000 transmission electron microscope (Hitachi, Tokyo, Japan) operated at 75 kV.

Flotation analysis. Flotation analysis was performed as described previously with modifications (21). Briefly, virus-infected cells were washed and scraped into PBS containing 0.9 mM CaCl₂ and 0.49 mM MgCl₂. Cells were pelleted by centrifugation for 2 min at $500 \times g$ and resuspended in 1.2 ml of lysis buffer (20 mM Tris-HCl [pH 8.0], 250 mM sucrose, 1 mM MgCl₂, and 1 mM CaCl₂) containing protease inhibitors at final concentrations of 1.04 mM aminoethylbenzene sulfonyl fluoride, 0.8 μ M aprotinin, 20 μ M leupeptin, 40 μ M bestatin, 15 μ M pepstatin A, and 14 μ M E-64. Cells were then disrupted by repeated passage (40 times) through a 22-gauge needle. Unbroken cells and nuclei were

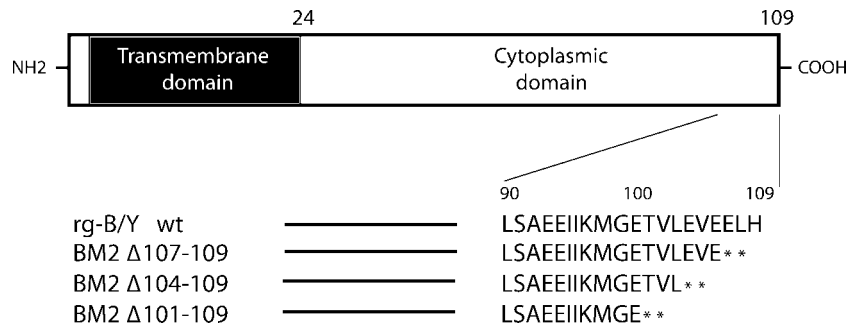


FIG. 1. Schematic diagram of wild-type and mutant BM2 proteins carrying a carboxyl-terminal truncation in the BM2 cytoplasmic domain. Amino acid sequence of the BM2 cytoplasmic domain (residues 90 to 109) is shown. The asterisk indicates a stop codon.

removed by centrifugation at $1,000 \times g$ for 10 min at 4°C. Postnuclear supernatants (1.2 ml) were dispersed into 1.2 ml of 60% (wt/wt) OptiPrep (AXIS-SHIELD PoC AS, Oslo, Norway) in 20 mM Tris-HCl (pH 8.0) plus protease inhibitors, placed at the bottom of the tube, and then overlaid with a gradient series of 25%, 20%, 15%, and 10% OptiPrep layers containing 20 mM Tris-HCl (pH 8.0), 125 mM sucrose, 1 mM MgCl₂, 1 mM CaCl₂, and protease inhibitors. The gradient was centrifuged for 14 h at $59,200 \times g$ using an SW-55Ti rotor in a Beckman ultracentrifuge. Samples (400 μl) of the gradient were collected from bottom to top and directly subjected to SDS-PAGE.

RESULTS

Generation of influenza B virus mutants carrying the truncated cytoplasmic domain of the BM2 protein by reverse genetics. To explore the functional role of the cytoplasmic domain of the BM2 protein in the replication cycle of influenza B virus, we constructed cDNAs encoding BM2 proteins with the cytoplasmic domain truncated at three (BM2Δ107-109), six (BM2Δ104-109), or nine (BM2Δ101-109) amino acids from the carboxyl-terminal end (Fig. 1). Influenza B viruses possessing these truncated BM2 proteins were generated by reverse genetics based on the backbone of B/Yamagata virus genes using eight Pol I plasmids encoding genomic RNAs and four protein expression plasmids (PB1, PB2, PA, and NP) as described previously (14). The resultant BM2Δ107-109 mutant was obtained with efficiency as high as that of the wild-type virus (rg-B/Y wt) from plasmid-transfected 293T cells, whereas the other two mutants, the BM2Δ104-109 and BM2Δ101-109 mutants, were not rescued (data not shown). These 2 mutants were recovered when our 12-plasmid system was supplemented with the wild-type BM2 expression plasmid as mentioned above. Rescued mutants in the culture supernatant were then amplified in the MDCK cell line, CK/BM2, which is able to constitutively express the wild-type BM2 protein for preparation of working stock viruses. Titers of virus stocks were determined by a plaque assay with CK/BM2 cells, and all mutants showed titers similar to that of rg-B/Y wt ($\sim 1 \times 10^8$ PFU/ml). By sequencing RNA segment 7 of recovered mutants, we confirmed no reversion and no additional mutations in the segment.

Synthesis and localization of BM2 proteins in cells infected with BM2 deletion mutants. To examine whether BM2 protein expression was affected by truncation of the cytoplasmic domain, MDCK cells were infected with mutants and synthesis of the truncated BM2 proteins was analyzed by Western blotting using anti-BM2 antibody. All truncated BM2 proteins were found with the expected molecular sizes, although migration

ratios of the BM2Δ104-109 and BM2Δ101-109 proteins did not differ significantly from each other (Fig. 2A). Expression levels of mutant proteins were estimated using a CS analyzer as described in Materials and Methods. The BM2Δ107-109 protein was expressed at levels equivalent to that of the wild-type BM2 protein, whereas BM2Δ104-109 and BM2Δ101-109 showed lower expression levels of 57% and 54%, respectively. These differences were not reflected in the efficacy of virus recovery and growth, as shown below.

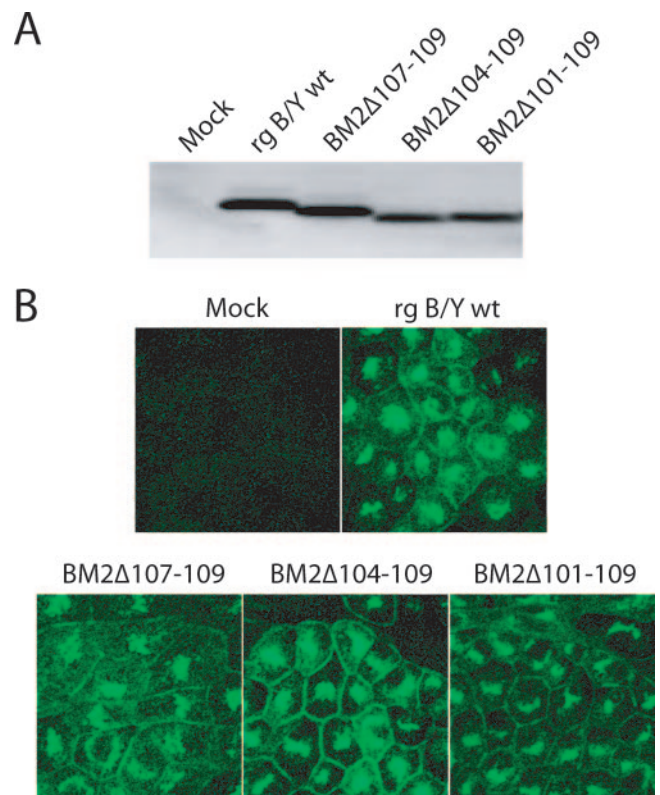


FIG. 2. Detection of the BM2 protein in BM2 truncation mutant virus-infected MDCK cells. (A) MDCK cells were infected with rg-B/Y wt and BM2stop viruses. At 10 h p.i., the BM2 protein in cell lysates was detected by Western blotting using anti-BM2 antibody. Quantitation of BM2 proteins was performed using an ATTO CS analyzer, and percentages were determined relative to values obtained for the wild-type BM2 protein. (B) At 11 h p.i., infected cells were fixed and stained for indirect immunofluorescence using anti-BM2 antibody.

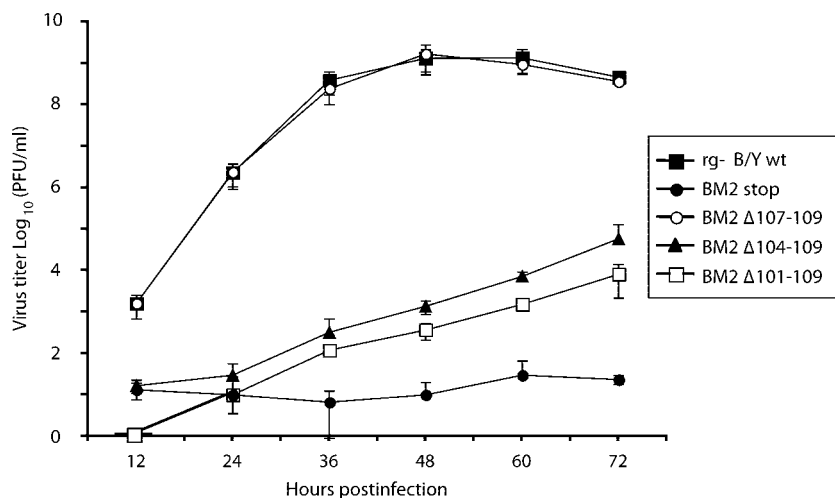


FIG. 3. Growth properties of BM2 truncation mutant viruses. MDCK cells were infected at an MOI of 0.001 PFU, and supernatants of infected cells were harvested at the indicated times. Titer of virus in the supernatant was determined by a plaque assay on CK/BM2 cells.

The BM2 protein is transported to the plasma membrane through the trans-Golgi network (47). To determine whether truncation in the cytoplasmic domain of the BM2 protein results in a failure of proper transport to the plasma membrane, localization of truncated BM2 proteins in mutant-infected MDCK cells was examined by IFA using anti-BM2 antibody (Fig. 2B). Truncated BM2 proteins were detected along the plasma membrane and at the Golgi apparatus of infected cells, and localization profiles were indistinguishable from that of the wild-type BM2 protein, indicating that truncation of nine amino acids from the carboxyl-terminal end of the BM2 protein does not significantly affect BM2 protein transport to the plasma membrane.

Growth properties of BM2 mutants in MDCK cells. We compared the kinetics of virus production in a multiple-step growth cycle among BM2 truncation mutants. MDCK cells were infected with virus at an MOI of 0.001, and titers of virus in the supernatant were measured by a plaque assay with CK/BM2 cells. rg-B/Y wt and the BM2 knockout mutant (BM2stop; see reference 14) were included for comparison of the growth properties of mutants. The BM2Δ107-109 mutant was indistinguishable in terms of both growth kinetics and maximum yield from rg-B/Y wt virus (Fig. 3). However, growth of the BM2Δ104-109 and BM2Δ101-109 mutants was extremely low, and the yield at 36 h p.i. was approximately 6 logs less than that of rg-B/Y wt, although growth was better than that of the BM2stop mutant. These results indicate that truncation of six or more amino acids at the carboxyl-terminal end caused great suppression of infectious virus production.

Viral components in the virion of BM2 mutant viruses. We have previously demonstrated using the BM2stop mutant that a lack of the BM2 protein severely blocks incorporation of vRNP complex into the virion (14). To determine whether truncation of the BM2 cytoplasmic domain affects incorporation of vRNP complex that can be traced by detection of the NP protein and a representative vRNA segment in the virion, MDCK cells were infected with mutant viruses at an MOI of 5 for 24 h. Progeny viruses released into the culture supernatant were purified and analyzed by SDS-PAGE followed by Co-

massie brilliant blue staining (Fig. 4A). Components of purified virions of mutants were compared with those of rg-B/Y wt and BM2stop viruses. In the virion of BM2Δ107-109 virus, which replicated normally (Fig. 3), the NP protein was incorporated at levels similar to those for rg-B/Y wt virus when total virion protein was normalized with the HA protein (Fig. 4B). In contrast, for BM2Δ104-109 and BM2Δ101-109 virions, mutants grew very poorly (Fig. 3) and incorporation of the NP protein into virions was dramatically decreased, to levels similar to that for the BM2stop mutant, although each of the truncated BM2 proteins was incorporated into the particles (Fig. 4B). Moreover, amounts of M1 proteins in the BM2Δ104-109 and BM2Δ101-109 mutants were found to be 67% and 27%, respectively, of those in rg-B/Y wt virus. To determine the relative amounts of vRNA incorporation into BM2 mutant virions, vRNA was extracted from purified virions and copy numbers of the HA gene as a representation of vRNA segments were quantified by real-time reverse transcription-PCR using HA gene-specific primers as previously described (14). Incorporation of vRNA of the BM2Δ107-109 mutant was detected to be at a similar level to that of rgB/Y wt, whereas vRNA levels of the BM2Δ104-109 and BM2Δ101-109 mutants were 1/3 and 1/5 of levels for rgB/Y wt, respectively (Fig. 4C). Taken together, these results suggest that deletion of six amino acids from the carboxyl-terminal end of the BM2 protein gives rise to a marked decrease in vRNP complex incorporation in the virion assembly process, so that production of infectious particles is extremely suppressed.

We also observed that BM2stop, BM2Δ104-109, and BM2Δ101-109 virions contained increased amounts of nonviral proteins (Fig. 4A). However, proteins detected with virions of mutants may have represented contaminants attached to the virion surface and/or cell membrane fragments. To explore this, virions were treated with trypsin in the presence or absence of 1% Triton X-100 and then analyzed by Coomassie brilliant blue staining (Fig. 4D, upper panel). A 44-kDa polypeptide was still detected in virions even after trypsin treatment, as were the internal virion proteins NP and M1 (Fig. 4D, lanes 4, 8, and 10), but not after treatment in the

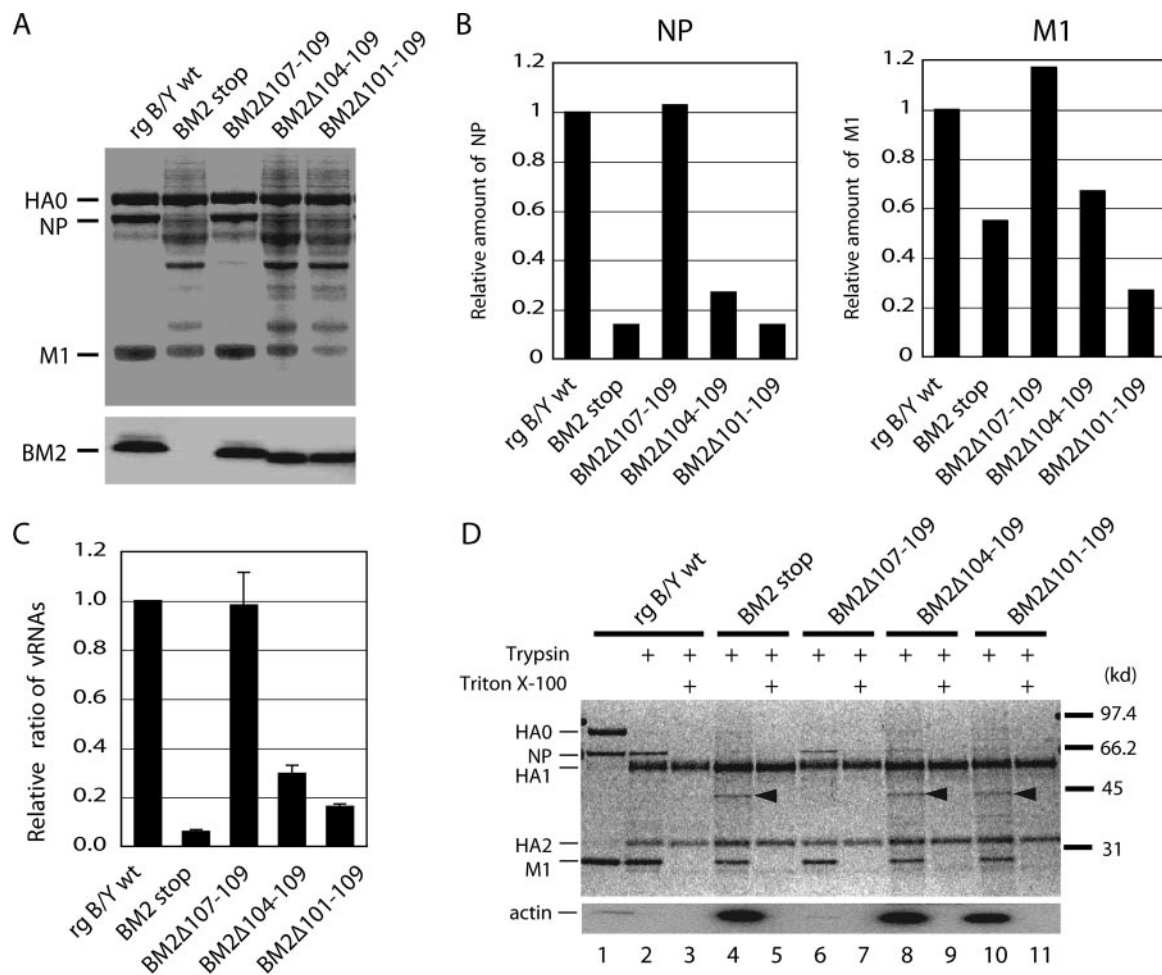


FIG. 4. Viral proteins and RNA segments in BM2 mutant virus particles. MDCK cells were infected at an MOI of 5 PFU, and virions were purified by centrifugation through 30% sucrose. (A) Protein composition of BM2 mutant and rg-B/Y wt virions. Purified virions produced by MDCK cells were analyzed by Coomassie brilliant blue staining (upper panel) and Western blotting using anti-BM2 antibody (lower panel). (B) Relative amounts of viral proteins. Viral proteins were quantified using an ATTO CS analyzer, and relative staining intensity of each protein was normalized to that of HA for each virus. (C) Relative ratio of HA genes between BM2 mutant and rg-B/Y wt virions. RNA was extracted from virions grown in MDCK cells and reverse transcribed, and the resultant cDNA was quantified using real-time PCR with primers directed to HA genes (see Materials and Methods). Mutant virions analyzed contained amounts of the HA protein equal to those of rg-B/Y wt virions. All samples were run in duplicate and repeated three times. Error bars represent standard errors of the mean. (D) Analysis of proteins in protease-treated virions. Purified virions were incubated at 25°C with 0.05 mg/ml trypsin in the absence or presence of 1% Triton X-100. AEBSF was then added to 4 mM, and each sample was analyzed by Coomassie brilliant blue staining (upper panel) and by Western blotting using antiactin antibody (lower panel). Arrowheads indicate cellular actin. Mobilities of molecular mass markers are shown on the right.

presence of Triton X-100 (Fig. 4D, lanes 5, 9, and 11), indicating that the 44-kDa polypeptide was incorporated into virions together with viral components. Influenza A virus viruses are known to incorporate cellular actin into virions (39), and this protein has a molecular mass of 42 kDa. As a result, we next examined whether the 44-kDa polypeptide really represented actin by Western blotting analysis using antiactin antibody. The data indicated that BM2stop, BM2Δ104-109, and BM2Δ101-109 virions contained increased amounts of actin (Fig. 4D, lower panel). Taken together, these data suggest that exclusion of actin from progeny virions is affected by truncations in the cytoplasmic domain of the BM2 protein.

Analysis of virions by electron microscopy. Previous studies of influenza A virus have shown that deletion of the cytoplasmic domains of the HA and NA glycoproteins and the M2

protein affects the size and shape of the virus particle, often resulting in formation of long filamentous structures (16, 17, 27). To determine whether the lack of BM2 and truncation of the BM2 cytoplasmic domain alters normal morphogenesis in B/Yamagata virus particles, MDCK cells were infected with mutant viruses at an MOI of 5 for 24 h and virions were purified and examined by negative-staining electron microscopy (Fig. 5). BM2Δ107-109 virions displayed a typical uniform spherical shape with a diameter similar to that of rg-B/Y wt virions (Fig. 5). However, BM2Δ104-109, BM2Δ101-109, and BM2stop virions were enlarged and either spherical or amorphous (Fig. 5). These results suggest that the failure of vRNP complex and M1 incorporation caused by the truncation or lack of the BM2 protein exerts profound effects on virion morphogenesis.

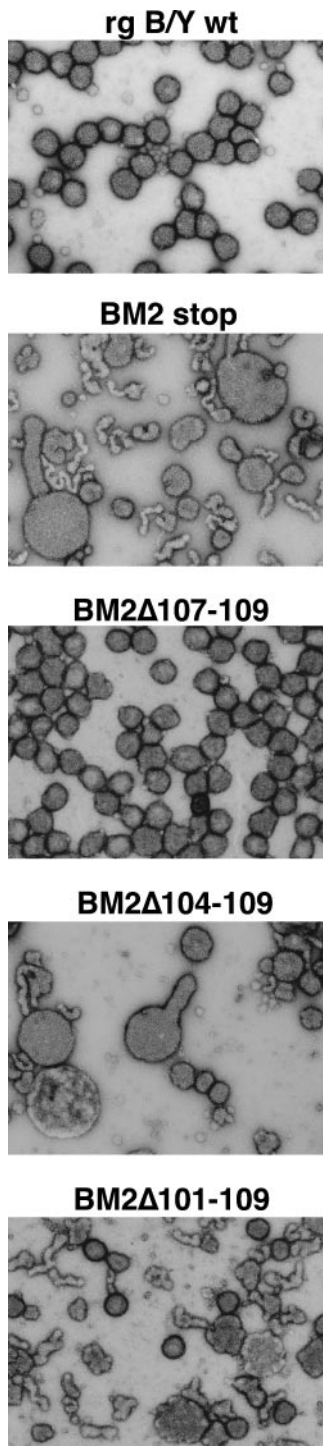


FIG. 5. Morphologies of mutants observed by using negative-staining electron microscopy. Virions were grown in MDCK cells and purified by centrifugation through 30% sucrose. Virions were negatively stained with 1% uranylacetate and observed by using transmission electron microscopy. Bar, 200 nm.

Truncated BM2 protein does not support intact infectious virus production. Since deletion of six amino acids from the carboxyl-terminal end of the BM2 protein was not tolerated for normal virion assembly or production of infectious virus, we

focused on the BM2 Δ 104-109 mutant for further characterization of the BM2 protein in the present study. BM2 knockout virus was able to grow normally when the wild-type BM2 protein was supplemented in *trans* by host cells expressing BM2 (14). We therefore examined whether growth of BM2 knockout virus could be regained by *trans* supplementation of the truncated BM2 protein. To this end, the growth properties of rg-B/Y wt, BM2stop, and BM2 Δ 104-109 viruses were examined in a cell line expressing wild-type BM2 or BM2 Δ 104-109. In the presence of supplemental wild-type BM2 protein, growth of BM2stop and BM2 Δ 104-109 viruses was restored to levels similar to that seen for rg-B/Y wt virus (Fig. 6A). The resulting virion also contained amounts of NP and M1 proteins comparable to those in rg-B/Y wt virus (Fig. 6B, lanes 2 and 3). Conversely, *trans* supplementation of the BM2 Δ 104-109 protein did not accelerate the growth of BM2stop and BM2 Δ 104-109 viruses (Fig. 6A), and virions failed to incorporate vRNP complex, although the truncated BM2 protein was incorporated into virions (Fig. 6B, lanes 5 and 6). These findings support the notion that the cytoplasmic domain of the BM2 protein plays a substantial role in packaging of vRNP complex during the assembly process and in the production of infectious virus.

Membrane association of M1 was greatly suppressed in the presence of the truncated BM2 protein. We have previously reported that BM2 increases the membrane association of M1 (14). To test whether truncation of the carboxyl-terminal region of BM2 alters the membrane association of M1, virus-infected cells at 11 h p.i. were examined by iodixanol flotation centrifugation analysis followed by Western blotting using anti-B/Yamagata and anti-BM2 antibodies. In this experiment, the majority of membrane sedimented in fractions 6 to 10. In cells infected with rg-B/Y wt, the majority of M1 (74%) sedimented in membrane fractions together with the integral transmembrane proteins, HA and BM2 (Fig. 7A, B, and D, upper row of each panel). In cells infected with the BM2 Δ 104-109 mutant, on the other hand, only small amounts of M1 floated up with membranes while the majority (71%) remained in fractions 1 to 5, containing the majority of soluble NP, although distributions of HA, NP, and BM2 resembled those of rg-B/Y wt (Fig. 7). Those distribution profiles were essentially identical to that of the BM2stop mutant. These results strongly suggest that the membrane association of M1 was affected by the six-amino-acid truncation of the carboxyl-terminal end of BM2, resulting in a failure to package vRNP complex into virions.

Characterization of influenza B virus mutants with alanine substitutions in the cytoplasmic domain. Since truncation of the carboxyl-terminal end of the BM2 protein affected normal incorporation of vRNP complex into virions (Fig. 4), this region can be postulated to contain an effector domain of the BM2 protein. To address this issue, we generated a series of alanine-scanning substitution mutants encoding a BM2 protein with a block of three adjacent amino acids changed to alanine, spanning amino acids 86 to 109 (Fig. 8), and amplified the mutants using the same methods used for recovery of the BM2 deletion mutant. Growth properties of these mutants in MDCK cells are shown in Fig. 9A. The BM2 104-106A and BM2 107-109A mutant viruses exhibited growth kinetics indistinguishable from that of rg-B/Y wt virus. The BM2 98-100A and BM2 101-103A mutants revealed slightly delayed virus

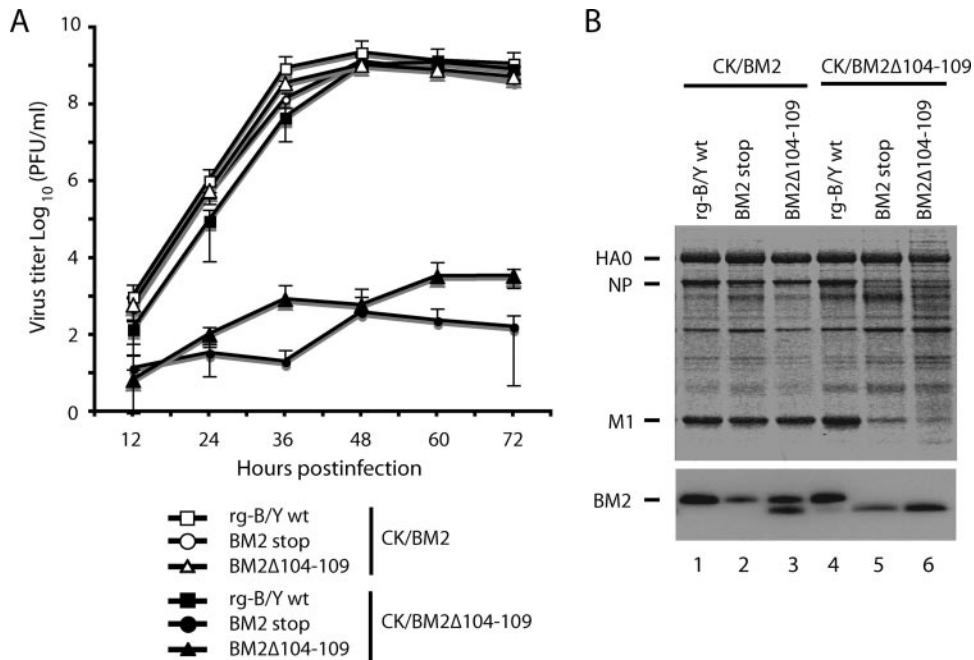


FIG. 6. The BM2 Δ 104-109 mutant protein did not support intact infectious virus production. (A) Multiple-step growth curve of rg-B/Y wt, BM2stop, and BM2 Δ 104-109 viruses. CK/BM2 and CK/BM2 Δ 104-109 cells were infected at an MOI of 0.001 PFU, and supernatants of infected cells were harvested at the indicated times. Titer of virus in the supernatant was determined by a plaque assay on CK/BM2 cells. (B) Protein composition of rg-B/Y wt, BM2stop, and BM2 Δ 104-109 virions was analyzed by Coomassie brilliant blue staining (upper panel) and Western blotting using anti-BM2 antibody (lower panel).

production but reached titers similar to that of rg-B/Y wt virus at 60 h p.i. In contrast, BM2 89-91A, BM2 93-94A, and BM2 95-97A mutants did not display substantial growth, similar to the BM2stop mutant, and titers at 60 h p.i. were approximately 6 logs lower than that of rg-B/Y wt virus. Virions of these mutants, as expected, contained significantly reduced levels of NP and M1, although substitution BM2 mutant proteins were incorporated into the particles (Fig. 9B). The mutant BM2 86-88A had a titer higher than that of the BM2stop, BM2 89-91A, BM2 93-94A, and BM2 95-97A mutants but definitely lower than those of the rest of the mutants and rg-B/Y wt. Virus particles for this mutant also contained smaller amounts of NP. When the amounts of the HA protein from purified BM2stop, BM2 86-88A, BM2 89-91A, BM2 93-94A, and BM2 95-97A mutants were normalized, the amount of the NP protein in BM2 86-88A virions was 1.3- to 2.5-fold larger than those of the others (data not shown). This slight increase in NP incorporation into BM2 86-88A virions is likely to reflect an increase in virus production. These results indicate that 12 amino acids of the carboxyl terminus are variable but the adjacent upstream 12 amino acids are required for efficient assembly of vRNP complex into the virion.

DISCUSSION

The influenza B virus BM2 protein has the longest cytoplasmic domain of the various influenza virus integral membrane proteins. Previous studies using reverse-genetics techniques for influenza B virus have shown that viruses encoding BM2 proteins with large deletions in the cytoplasmic domain do not grow substantially in cell culture and produce progeny viruses

lacking vRNP complex (14), suggesting that the BM2 cytoplasmic domain might play an important role during the life cycle of influenza B virus.

To further explore the role of the long cytoplasmic domain of BM2 in the life cycle of influenza B virus, we generated and characterized the phenotypes of influenza B viruses bearing deletions or alanine substitutions within the BM2 cytoplasmic domain. Influenza B viruses encoding the BM2 protein with a six-residue (BM2 Δ 104-109) or nine-residue (BM2 Δ 101-109) deletion at the carboxyl terminus produced viruses containing dramatically reduced vRNP complex, as seen with BM2 knockout mutants, although intracellular transport was indistinguishable from that of the wild-type BM2 protein and incorporation into virus particles was achieved. Similarly, dramatic reductions of NP incorporation were also observed for virus particles of BM2 mutants (BM2 86-88A, BM2 89-91A, BM2 93-94A, and BM2 95-97A) with three consecutive alanine substitutions in the region from position 86 to 97. These data were further supported by observations that BM2 knockout virus was able to replicate successfully in cell culture by *trans* supplementation of the wild-type BM2 protein but not by *trans* supplementation of the truncated BM2 protein. Taken together, these findings indicate that the cytoplasmic domain of BM2 plays a crucial role for production of infectious virus particles. Similar evidence has recently been obtained for influenza A virus, where efficient production of infectious virus depends on the presence of the cytoplasmic domain of the M2 protein (16, 25).

BM2 and influenza A virus M2 have been shown to possess similar H⁺ ion channel activity (36). The ion channel is considered necessary for efficient vRNP uncoating during viral

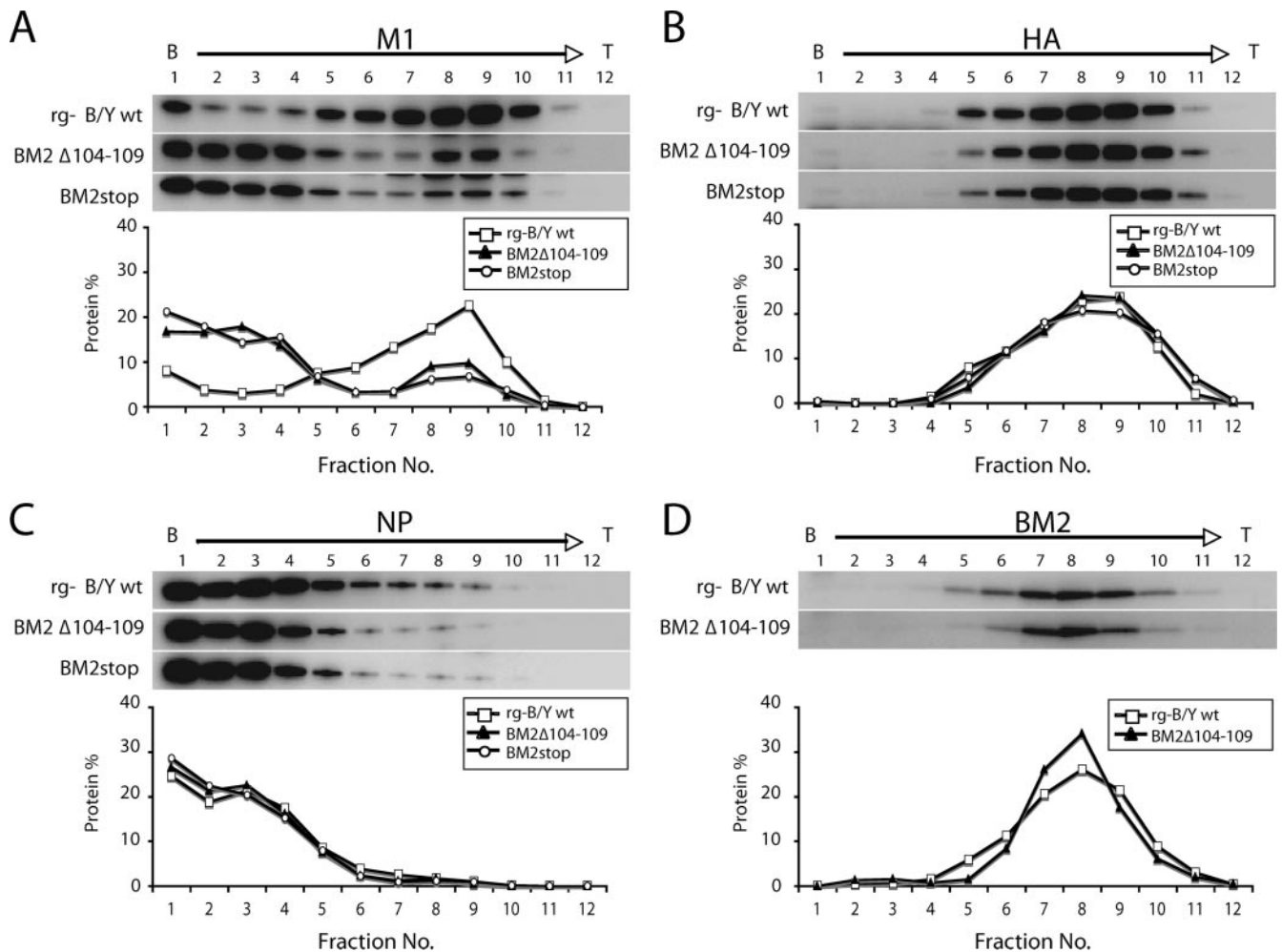


FIG. 7. Membrane association of viral proteins in virus-infected cells. MDCK cells were infected with rg-B/Y wt, BM2Δ104-109, and BM2stop viruses. At 11 h p.i., infected cells were lysed and postnuclear fractions were subjected to equilibrium centrifugation as described in Materials and Methods. M1 (A), HA (B), NP (C), and BM2 (D) proteins in all fractions collected from top (T) to bottom (B) were analyzed by Western blotting using anti-B/Yamagata and anti-BM2 antibodies (upper panels). Viral proteins were quantified using an ATTO CS analyzer. The amount of viral protein in each fraction is expressed as a percentage of the total amount in all fractions (lower panels).

entry (13). The transmembrane domain of influenza A virus M2 and influenza B virus BM2 contains consensus peptide motifs (19-HXXXW-23) involved in ion channel activity. Electrophysiological studies of BM2 in *Xenopus* oocytes and mammalian cells have demonstrated that substitutions of Cys at positions His19 and Trp23 result in reduced ion channel activity (28). However, the role of the cytoplasmic domain of the BM2 protein in ion channel activity has not been determined. In the case of influenza A virus, M2 molecules with truncated cytoplasmic domains display significantly reduced ion channel activity, indicating that the cytoplasmic domain is involved in the function of ion channel activity (45). If this is also the case for BM2, truncation of the cytoplasmic domain may affect ion channel activity. Thus, reduced ion channel activity of BM2 may represent one of the reasons for decreased virus infectivity. Using reverse-genetics techniques, we have observed that influenza B virus with amino acid changes at positions 19 and 23, causing defective ion channel activity, grows more slowly than wild-type virus in the multiple-step growth cycle but pro-

duces infectious progeny viruses with a maximum yield similar to that of wild-type virus (M. Obuchi, M. Imai, and T. Odagiri, unpublished observations). For the influenza A virus M2 protein, Watanabe et al. (48) suggested that ion channel activity is dispensable for growth in tissue culture, whereas Takeda et al. (43) argued against those findings following analysis of amantadine sensitivity and concluded that ion channel activity contributes to efficient virus replication. These studies suggest that H⁺ ion channel activity is important for efficient growth of both influenza A and influenza B viruses but that activity may not be absolutely essential for virus viability in tissue culture.

We also found that truncation of the BM2 cytoplasmic domain significantly affects not only incorporation of vRNP into virions but also incorporation of M1. For influenza A virus M2, however, truncation of the cytoplasmic domain does not affect packaging of M1 into virions (16, 25). The reasons for this difference remain unclear, but we speculate that differences in the nature of protein-protein interactions for the vRNP-M1 complex between influenza A and influenza B viruses are re-

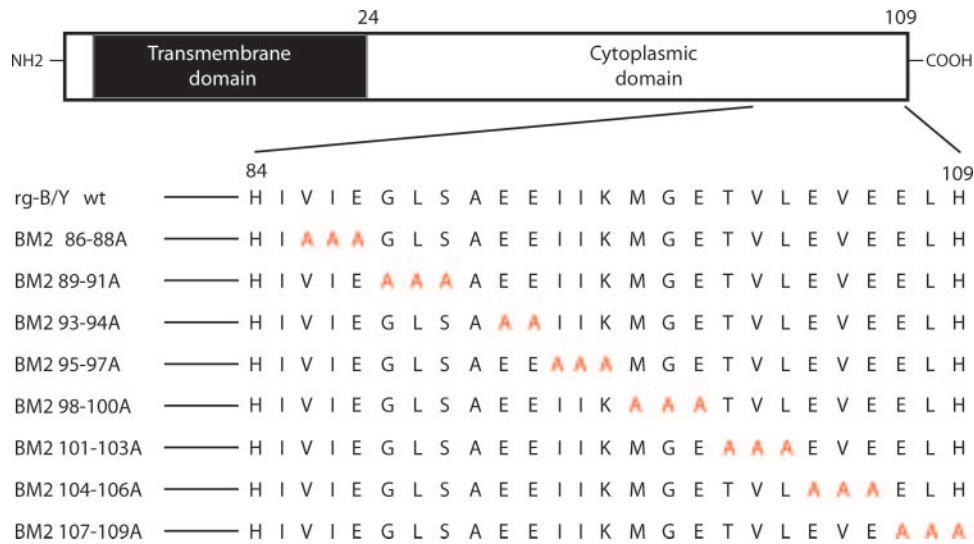


FIG. 8. Schematic diagram of wild-type and mutant BM2 proteins with alanine substitutions in the BM2 cytoplasmic domain. Amino acid sequence of the BM2 cytoplasmic domain (residues 84 to 109) is shown. Mutated amino acid residues are indicated in red for each BM2 mutant.

sponsible. Interestingly, complex formation among vRNP, M1, and NEP/NS2 of influenza B virus has been shown to differ from that with influenza A virus (15). Further characterization of interactions among viral components is required to elucidate the final steps of virus assembly.

The M1 protein is a multifunctional protein that plays central roles in virus assembly and budding. When expressed in cells, M1 can induce the formation and release of M1-containing particles into extracellular medium without the aid of other viral proteins (10, 19). The protein also exhibits lipid-binding properties (3, 11, 38) and associates tightly with cell membranes to form the inner surface of the lipid bilayer of the envelope (1, 6, 18, 51). Moreover, M1 interacts both with vRNP to mediate nuclear export and prevent nuclear reentry of vRNP (23, 50) and with cytoplasmic domains of the HA and NA proteins on the plasma membrane (1, 6, 17, 53). M1 thus appears to coordinate particle formation of influenza virus by forming a bridge between the lipid envelope and various virus components. We have previously shown that membrane association of influenza B virus M1 is highly influenced by the presence or absence of BM2 on the membrane (14). In the present study, we also observed that truncation of the BM2 cytoplasmic domain causes decreased membrane association of M1 by flotation gradient analysis, suggesting that the cytoplasmic domain of BM2 interacts with the M1 protein and increases the membrane-binding affinity of the M1 protein (Fig. 7). Support for this notion is provided by previous experiments in which BM2 was coprecipitated with M1 when detergent-disrupted virions were immunoprecipitated using anti-M1 antibody and by the finding that in experiments of virion fractionation, BM2 always coexists with M1 in the same fractions but is not present with HA and vRNP without M1 (32). Based on these findings, it is tempting to speculate that a major contribution of this M1-BM2 interaction is to capture M1-vRNP complex at the virion budding site during virus assembly. Interestingly, small amounts of M1 in BM2 mutant virions remained detectable when vRNP incorporation was drastically

reduced (Fig. 4 and 9). We therefore postulate that two populations of M1 molecules exist within virions: vRNP-associated M1 and vRNP-free M1. The cytoplasmic domain of the BM2 protein may selectively bind to vRNP-associated M1 but not to vRNP-free M1, since the number of BM2 molecules within infected cells is much lower than that of M1 molecules.

In addition to vRNP packaging, M1-BM2 interaction is likely to control the morphology of virus budding. Influenza B viruses with BM2 proteins containing altered cytoplasmic domains display a wide range of sizes and shapes relative to those of wild-type viruses, as determined by negative-staining electron microscopy (Fig. 5). Initially, M1 was thought to be the major driving force in virus budding (10, 19), although a subsequent study found that M1 of influenza A virus was not released efficiently into culture medium when M1 was expressed in the absence of other viral proteins, suggesting that M1 may not be required for virus budding (4). However, M1 is known to modulate virus particle morphology (2, 5). We therefore hypothesize that changes in the strength of M1-membrane interaction may down-regulate the efficiency of virus release, leading to significant changes in virus particle morphology. Our study using BM2 mutants suggested the importance of M1-BM2 interactions in the morphological features and assembly of influenza B virus. We also observed that BM2stop, BM2 Δ 104-109, and BM2 Δ 101-109 mutant particles with altered morphology had a much greater content of cellular actin than spherical virions, such as wild-type and BM2 Δ 107-109 viruses. The cellular cytoskeleton has been proposed to play an important role in bud formation and bud release of influenza virus. For influenza A virus, cellular actin and the actin-binding protein ezrin-radixin-moesin have been found in purified preparations of particles (39). Furthermore, disruption of the actin cytoskeleton inhibits the production of filamentous virions but not spherical virions, suggesting that formation depends on intact actin filaments (37, 42). Our data suggest that exclusion of a large proportion of actin from the viral budding site was impaired by deletion of the BM2 cytoplasmic domain. Efficient

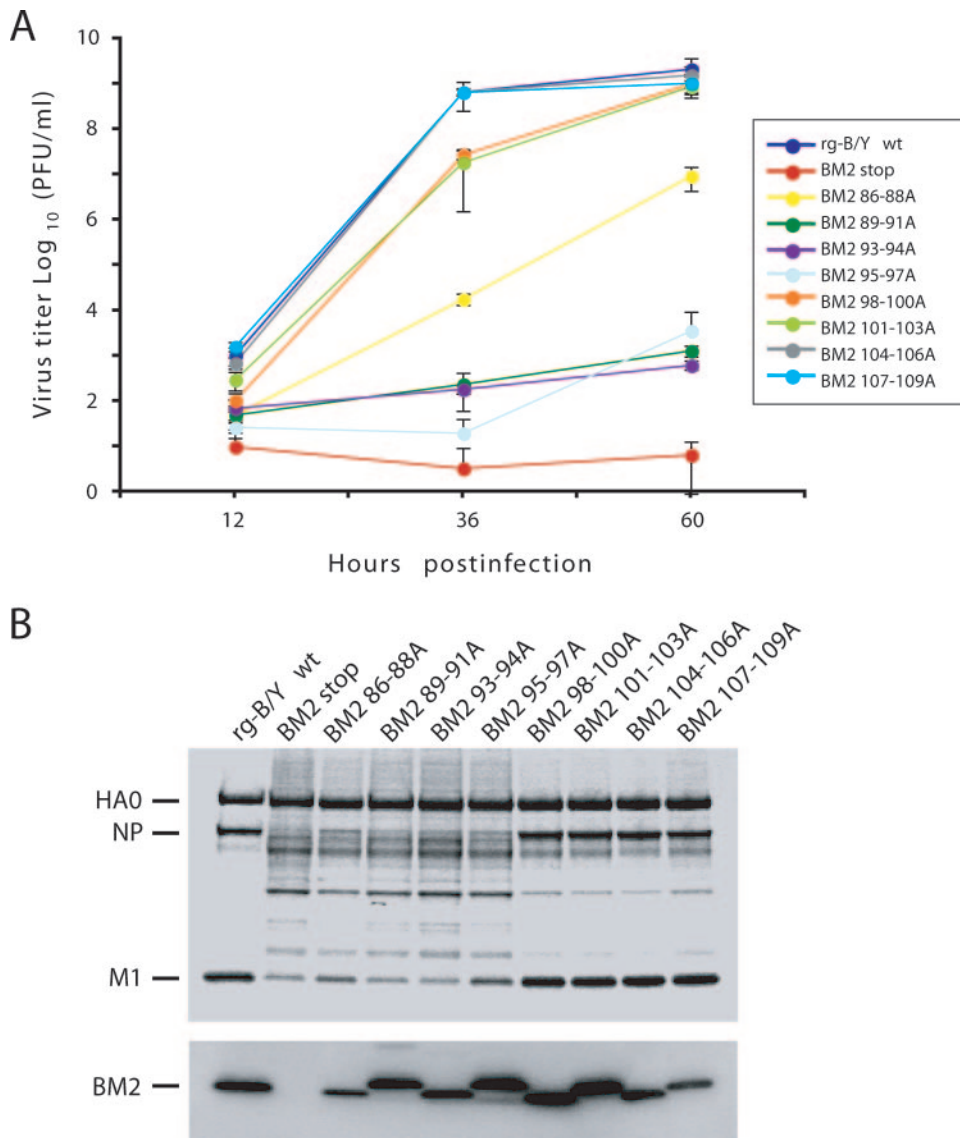


FIG. 9. Characterization of influenza B virus mutants with alanine substitutions in the cytoplasmic domain. (A) Multiple-step growth curve of BM2 mutant viruses. MDCK cells were infected at an MOI of 0.001 PFU, and culture medium was harvested at the indicated times. Virus yield of the supernatant was determined by plaque assay on CK/BM2 cells. (B) Protein composition of BM2 mutant virions. Proteins of purified viruses were analyzed by Coomassie brilliant blue staining (upper panel) and Western blotting using anti-BM2 antibody (lower panel).

exclusion of host proteins from virions might be involved in the maturation of spherical virus particles.

Previous studies have shown that sequences at both the 5' and 3' ends of the vRNA coding region are necessary for efficient incorporation of the vRNA segment into virions (8, 9, 20, 22, 29, 34, 49). We therefore cannot exclude the possibility that the mutations introduced into 5' coding ends of the M segment disrupt packaging signals, resulting in reduced viral replication. However, we found that when the wild-type BM2 protein was supplemented by host cells expressing BM2, the BM2 cytoplasmic domain-truncated virus (BM2 Δ 104-109 mutant) exhibited growth kinetics indistinguishable from that of rg-B/Y wt virus, and no significant differences existed in the incorporation of the M1 protein between the mutant and wild-type viruses (Fig. 6). Consequently, we favor the interpretation

that viral infectivity was reduced as a result of truncation of the BM2 cytoplasmic domain.

Since truncation of the carboxyl-terminal three residues of BM2 (BM2 Δ 107-109) had no effect on production of infectious viruses but truncation of six or nine residues (BM2 Δ 104-109 or BM2 Δ 101-109) significantly impaired vRNP incorporation into particles, a six-amino-acid region encompassing residues 101 to 106 should contain an essential domain for production of infectious virus. However, we observed that neither alanine substitutions of residues 101 to 103 (BM2 101-103A) nor substitutions of residues 104 to 106 (BM2 104-106A) had any effect on infectious virus production. These observations suggest that the sequence in this region of the BM2 cytoplasmic domain is not critical for vRNP complex incorporation into virions but the actual length of the cytoplasmic domain might be important for fulfilling BM2 function.

Serial substitutions in the cytoplasmic domain of the BM2 protein indicated that a 12-amino-acid stretch between amino acids 86 and 97 cannot be replaced by other sequences to preserve the innate function of BM2 (Fig. 9). The region may contain effector signals for binding of the M1 protein, like the signal found with the M2 protein of influenza A virus (24). Another possibility is that this region has a secondary structure essential for the stability of the BM2 protein. Alternatively, since the BM2 protein has been shown to be modified by phosphorylation (32), a serine-to-alanine change at position 91 might result in the loss of a phosphorylation site, so the function of BM2 may be affected. However, phosphorylation of M2 in influenza A virus does not appear to be essential for virus replication (44). Further investigations in terms of these possibilities are needed.

In summary, the present study showed that the presence and maintenance of a long cytoplasmic domain in BM2 is important for the function of the BM2 protein in the membrane association of the M1 protein, incorporation of vRNP and M1 into virions, and particle morphology. Moreover, we have presented evidence that suggests the amino acid region from position 86 to position 97 in the BM2 cytoplasmic domain is a prerequisite for innate BM2 function. These findings greatly enhance our understanding of the molecular mechanisms underlying influenza virus assembly.

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