

Influenza B Virus BM2 Protein Is a Crucial Component for Incorporation of Viral Ribonucleoprotein Complex into Virions during Virus Assembly

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Influenza B virus contains four integral membrane proteins in its envelope. Of these, BM2 has recently been found to have ion channel activity and is considered to be a functional counterpart to influenza A virus M2, but the role of BM2 in the life cycle of influenza B virus remains unclear. In an effort to explore its function, a number of BM2 mutant viruses were generated by using a reverse genetics technique. The BM2 Δ ATG mutant virus synthesized BM2 at markedly lower levels but exhibited similar growth to wild-type (wt) virus. In contrast, the BM2 knockout virus, which did not produce BM2, did not grow substantially but was able to grow normally when BM2 was supplemented in *trans* by host cells expressing BM2. These results indicate that BM2 is a required component for the production of infectious viruses. In the one-step growth cycle, the BM2 knockout virus produced progeny viruses lacking viral ribonucleoprotein complex (vRNP). The inhibited incorporation of vRNP was regained by *trans*-supplementation of BM2. An immunofluorescence study of virus-infected cells revealed that distribution of hemagglutinin, nucleoprotein, and matrix (M1) protein of the BM2 knockout virus at the apical membrane did not differ from that of wt virus, whereas the sucrose gradient flotation assay revealed that the membrane association of M1 was greatly affected in the absence of BM2, resulting in a decrease of vRNP in membrane fractions. These results strongly suggest that BM2 functions to capture the M1-vRNP complex at the virion budding site during virus assembly.

Influenza A, B, and C viruses are enveloped viruses that assemble at the plasma membrane and bud from infected cells. Both influenza A and B viruses have eight single-stranded negative-sense RNA segments encapsidated with polymerase proteins and nucleoprotein (NP), forming a viral ribonucleoprotein complex (vRNP). The envelope contains three different membrane proteins, hemagglutinin (HA), neuraminidase (NA), and ion channel protein M2 in influenza A virus and NB in influenza B virus (20). The small proteins, M2 and NB, are transmembrane proteins which adopt an N-out C-in orientation (21, 35, 38) and are incorporated into virions as a relatively minor component (3, 37). On the other hand, in the case of influenza A virus, the most abundant product matrix (M1) protein has multiple functions and plays central roles in virus assembly. M1 interacts with vRNP in the nucleus to mediate nuclear export and to prevent nuclear reentry of vRNP (22, 34) and with the cytoplasmic tail of HA and NA on the plasma membrane to form the inner surface of the lipid bilayer of the envelope (1, 7, 18, 40). These interactions are thought to be essential for particle formation and virus release from infected cells.

Although both influenza A and B viruses possess similarities in the coding strategy of genome RNA segments and in the

function of viral proteins, there are some differences between these viruses. One of the differences is found in RNA segment 7 of influenza B virus, which encodes the BM2 protein in addition to encoding M1 by bicistronic mRNA (4, 12). The translational strategy of BM2 is quite unique. The initiation codon of BM2 overlaps the termination codon of M1 in an overlapping stop-start pentanucleotide, TAATG, at nucleotides 769 to 773 (15). BM2 synthesis is triggered by the termination of translation of M1, which is encoded in the upstream region of mRNA (15). Because the amino acid sequence of BM2 has been highly conserved for at least 60 years, from B/Lee/40 to B/Nagoya/20/99 (Influenza Sequence Database, <http://www.flu.lanl.gov>), and BM2 is detected in cells infected with all influenza B viruses examined to date (15, 27), BM2 is thought to play an important role(s) in the life cycle of influenza B virus.

The BM2 protein was previously shown to be a phosphoprotein that is synthesized in the late phase of infection and is incorporated into the virion (27). Recently, Paterson et al. (28) and our group (32) demonstrated by several distinct experimental approaches that BM2 is associated with cellular membranes as an oligomeric integral membrane protein. It was also noted that BM2 accumulated at the Golgi apparatus immediately after synthesis and was then transported to the plasma membrane through the *trans*-Golgi network and that the N-terminal half of BM2 (residues 2 to 50) was crucial for this transport (32). In addition, Mould et al. (24), by using an electrophysiological technique, indicated that BM2, like influenza A virus M2, has an ion channel activity to permit protons

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TABLE 1. Virus yield of transfectants recovered from 293T cells transfected with 8 polI/BY plasmids and 4 pCA/ORF plasmids^a

Transfectant virus	Virus yield (PFU/ml)
rg-B/Y wt	1.4×10^4
BM2 Δ ATG	3.5×10^4
BM2 Δ 2-23	ND ^b
BM2 Δ 24-50	ND
BM2 Δ 51-80	ND
BM2 Δ 81-109	ND

^a 293T cells were transfected with 12 plasmids for production of rg-B/Y wt virus or BM2 mutant viruses. The infectious particles in the supernatants were recovered at 48 hpt and titrated in MDCK cells.

^b ND, not detected.

influenza A and B viruses with the desired mutations by transfection of either 8 plasmids (13, 14) or 12 to 17 plasmids (9, 17, 25). We adopted a 12-plasmid system for the generation of infectious influenza B viruses. To establish a reverse genetics system for the B/Yamagata virus cDNA backbone, we constructed PolI plasmids that contain cDNAs for the full-length vRNAs of the B/Yamagata virus flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator. These PolI plasmids were cotransfected with four protein expression plasmids (PB1, PB2, PA, and NP) into 293T cells. Using this system, we attempted to generate a BM2 knockout virus designated BM2 Δ ATG, whose BM2 initiation codon ₇₇₁ATG was replaced with ₇₇₁ACC (Fig. 1, middle), and four BM2 deletion mutant viruses, BM2 Δ 2-23, BM2 Δ 24-50, BM2 Δ 51-80, and BM2 Δ 81-109, whose PolI plasmids contained cDNAs with deletions in the BM2 open reading frame (ORF), as described previously (32). To recover the transfectants, the supernatant of plasmid-transfected 293T cells was harvested at 48 hpt. Table 1 shows the yields of transfectant produced in the supernatant. The BM2 Δ ATG mutant virus was recovered at similar titers as for the transfectants of B/Yamagata (rg-B/Y) wt virus. In contrast, the four BM2 deletion mutant viruses were not recovered at all. By sequencing RNA segment 7 of the recovered BM2 Δ ATG mutant virus, we confirmed no reversion from ₇₇₁ACC to the original ₇₇₁ATG and no additional mutations in the segment. This result suggests that BM2 synthesis occurs in cells infected with the BM2 Δ ATG mutant virus despite the absence of the ATG initiation codon in the BM2 ORF. To confirm this, BM2 Δ ATG mutant virus and rg-B/Y wt virus (control) were infected into MDCK cells and BM2 synthesis was investigated by Western blotting and IFA with anti-BM2 antibody (27). The BM2 signal for the mutant virus was not clearly detected by Western blotting (Fig. 2A), but IFA confirmed weak signals at the Golgi apparatus of infected cells (Fig. 2B). Moreover, extremely small amounts of BM2 were detected in purified virions, and quantitative comparison between the BM2 Δ ATG mutant and rg-B/Y wt virions indicated that the BM2 content normalized to the HA (HA1 plus HA2) content was 31% of that in rg-B/Y wt virus (Fig. 2C). It should be noted that the ratios of NP to HA of the mutant virus were also decreased slightly compared to those of rg-B/Y wt virus (Fig. 2C).

When the multiple-step growth profile of BM2 Δ ATG mutant virus was compared to that of rg-B/Y wt virus, the mutant virus revealed slightly delayed virus production, but its maximum yield was not significantly different from that of rg-B/Y wt

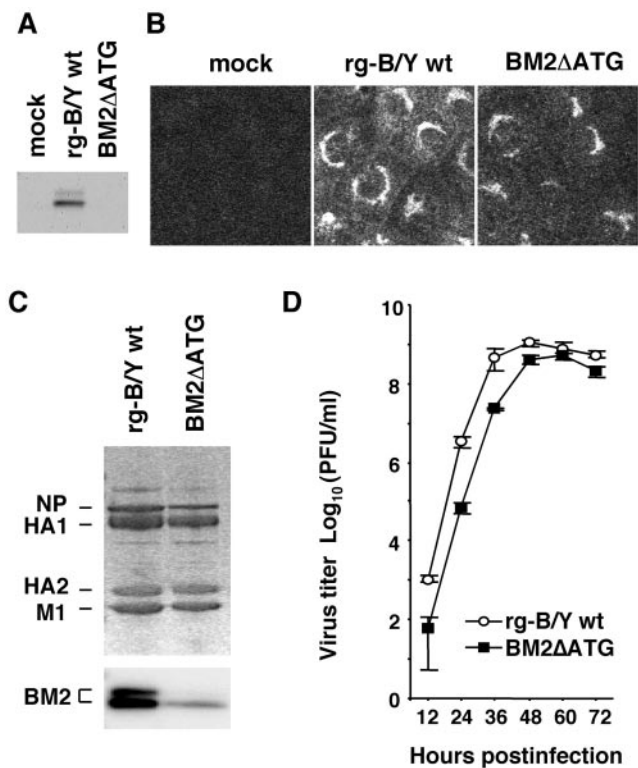


FIG. 2. Characterizations of BM2 Δ ATG mutant virus. (A) MDCK cells were infected with rg-B/Y wt and BM2 Δ ATG viruses. At 11 hpi, BM2 protein in the cell lysates was detected by Western blotting with anti-BM2 antibody. (B) At 7 hpi, cells were fixed and BM2 was detected with anti-BM2 antibodies by IFA. (C) Protein composition of BM2 Δ ATG virions. Proteins of purified viruses were analyzed by Coomassie brilliant blue staining (upper panel) and Western blotting with anti-BM2 antibody (lower panel). (D) Multiple-step growth curve of BM2 Δ ATG mutant viruses. MDCK cells were infected at an MOI of 0.001 PFU, and culture media were harvested at the indicated times postinfection. The virus yield of the supernatant was determined by plaque assay on MDCK cells.

virus (Fig. 2D). Taken together, these results clearly indicate that small amounts of BM2 can facilitate the production of infectious virus.

Rescue of BM2 knockout virus by supplementing BM2 from host cells stably expressing BM2. Because BM2 Δ ATG mutant virus synthesized BM2 at markedly low levels and grew as efficiently as rg-B/Y wt virus, we attempted to produce a genuine BM2 knockout virus. To accomplish this, the PolI plasmid BM2stop, which contained three consecutive stop codons downstream of the ₇₇₁ACC replacement found in BM2 Δ ATG and two additional mutations (A₈₃₁G and T₈₃₂C), was constructed (Fig. 1, bottom) and transfected into 293T cells in the same manner as for the 12-plasmid system. As expected, no infectious virus was recovered from the supernatant of plasmid-transfected cells (data not shown), thus suggesting that *trans*-supplementation of BM2 would be necessary for recovery of the mutant. We therefore prepared an MDCK cell line, CK/BM2, that was able to constitutively express BM2 protein, as described in Materials and Methods. In CK/BM2 cells, expression levels and localization of BM2 were similar to those in virus-infected cells at 10 h postinfection (hpi) (Fig. 3).

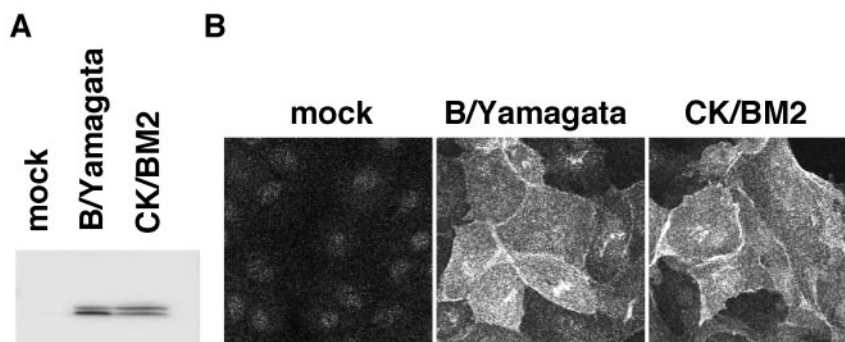


FIG. 3. Stable expression of BM2 proteins in MDCK cells. (A) Western blotting analysis with anti-BM2 antibody. Lanes: mock, uninfected cells; B/Yamagata, virus-infected cells; CK/BM2, constitutive BM2-expressing MDCK cell line. (B) Detection of BM2 synthesized in CK/BM2 cells (CK/BM2) at 10 hpi and in virus-infected cells (B/Yamagata) by IFA with the anti-BM2 antibody. Mock, uninfected cells fixed 10 h after incubation.

For the rescue of BM2stop mutant virus, 12 plasmids as mentioned above with the BM2 expression plasmid, pCAGGSBM2 (27), were transfected into 293T cells. The supernatant harvested at 48 hpt was then inoculated into CK/BM2 cells to detect transfectants. BM2stop mutant virus was recovered efficiently with a titer similar to that of the rg-B/Y wt and BM2 Δ ATG viruses (7.6×10^4 , 4.4×10^4 , and 1.1×10^4 PFU/ml, respectively). To confirm whether rescued BM2stop mutant virus lacked the synthesis of BM2, mutant virus-infected MDCK cells were subjected to Western blotting and IFA. Unlike BM2 Δ ATG mutant virus, BM2 synthesis was not detected for BM2stop mutant virus in either analysis (Fig. 4), indicating that BM2stop mutant virus was a BM2 knockout virus.

We next compared the kinetics of virus production in multiple-step growth cycles between BM2stop mutant and rg-B/Y wt viruses. In the absence of *trans*-supplementation of BM2 in MDCK cells, the mutant virus did not grow significantly (Fig. 5A), whereas in the presence of *trans*-supplementation of BM2 from CK/BM2 cells, the mutant virus regained its ability to grow and exhibited indistinguishable growth kinetics and maximum yield compared to rg-B/Y wt virus (Fig. 5B). These results strongly indicate that BM2 is a necessary component for the production of infectious virus.

Synthesis and localization of viral proteins in cells infected with BM2 knockout virus. To examine the synthesis and localization of virus proteins in MDCK cells, the cells were infected

with BM2stop mutant and rg-B/Y wt viruses. Figure 6A shows the synthesis of viral proteins in MDCK cells every 2 hpi, as detected by Western blotting with anti-BM2 or anti-B/Yamagata virus antibodies (27). BM2 of rg-B/Y wt virus was detected from 6 hpi but was not detected from BM2stop mutant virus throughout infection, which is consistent with the results shown in Fig. 4. On the other hand, the synthesis of major viral proteins, HA, NP, and M1, in the BM2stop mutant virus was similar to that of rg-B/Y wt virus, thus indicating that the synthesis of major viral proteins was not influenced by the deletion of BM2 synthesis.

It was previously demonstrated that BM2 is transported to the plasma membrane through the *trans*-Golgi network (32). Therefore, if BM2 interacted with major viral proteins in the cytoplasm of infected cells, the lack of BM2 may affect the intracellular transport of major viral proteins to the virion budding site at the apical membrane. Accordingly, we observed the distribution of HA, NP, and M1 at the apical membrane in an XZ section by using a confocal microscope. Consistent with the above findings, no BM2 signal was detected for the BM2stop mutant virus at the apical membrane, but BM2 was detected for the rg-B/Y wt virus, as reported by Paterson et al. (28) (Fig. 6B). HA protein was observed along the apical membrane (Fig. 6B), and the NP and M1 proteins were detected near the apical membrane containing HA (Fig. 6C). The distributions of these major viral proteins were quite similar between the BM2stop mutant and rg-B/Y wt viruses.

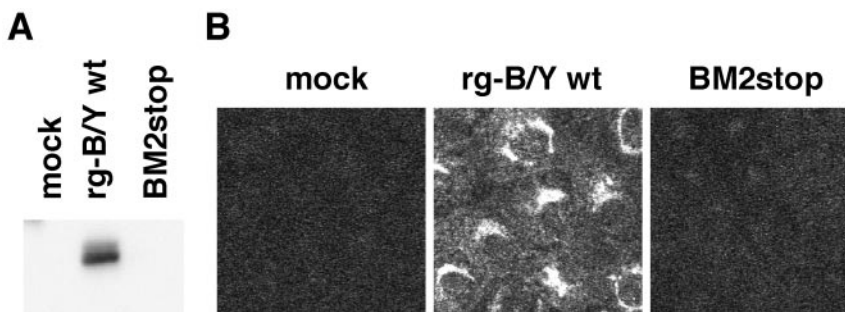


FIG. 4. Detection of BM2 protein in BM2stop mutant virus-infected MDCK cells. (A) MDCK cells were infected with rg-B/Y wt and BM2stop viruses. At 11 hpi, BM2 protein in the cell lysates was detected by Western blotting with anti-BM2 antibody. (B) At 7 hpi, cells were fixed and BM2 was detected by IFA with anti-BM2 antibodies. mock, uninfected cells.

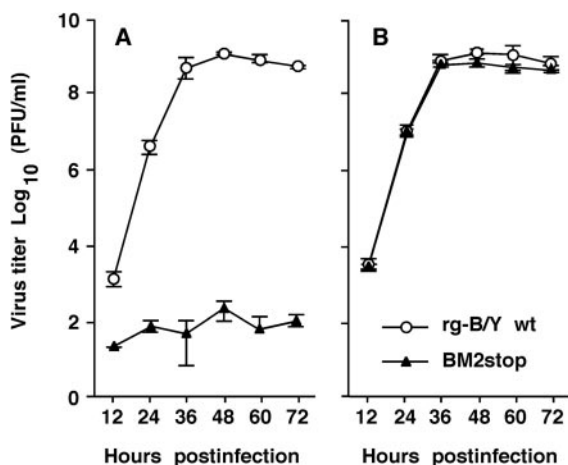


FIG. 5. Growth properties of BM2stop mutant virus. MDCK (A) and CK/BM2 (B) cells were infected at an MOI of 0.001 PFU, and the supernatants of infected cells were harvested at the indicated times postinfection. Virus titers in the supernatant were determined by plaque assay on CK/BM2 cells.

Viral components in virions of BM2 knockout virus. It is of interest to know why the BM2stop mutant virus did not grow in the absence of BM2 and how this mutant virus regained its ability to grow by supplementation of BM2. To clarify these issues, the BM2stop mutant and rg-B/Y wt viruses were infected into MDCK and CK/BM2 cells at a multiplicity of infection (MOI) of 5 PFU per cell, and progeny viruses released into the culture supernatant were purified and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 7). In the virion of the mutant virus produced by MDCK cells, the BM2 was not detected at all and NP was not clearly detected in the virions. Moreover, the content of M1 normal-

ized with that of HA was found to be less than half of that in rg-B/Y wt virus (Fig. 7B). In contrast, in the virion of mutant viruses produced by CK/BM2 cells, NP and M1 were recovered at about 80 and 90%, respectively, of the levels in rg-B/Y wt virus (Fig. 7A and B). These results indicate that the incorporation of M1 and NP into virions was highly affected in the absence of BM2. Interestingly, comparable amounts of BM2 supplied by CK/BM2 cells were also incorporated into the virions.

Since NP, which constitutes the backbone of the vRNP, was reduced in the mutant virions, we speculated that BM2 is required for the packaging of vRNP. Further studies were therefore conducted to determine whether incorporation of vRNA into virions was affected by the absence of BM2 expression. RNA was extracted from the purified BM2stop mutant and rg-B/Y wt virions grown in MDCK cells. The mutant virions used in this experiment had an amount of HA proteins equal to that of rgB/Y wt virions. After extraction, vRNA was reverse transcribed and the resultant cDNA was analyzed by real-time PCR with primers directed to the PB1, HA, and M genes (see Materials and Methods). Copy numbers of the vRNA detected in the mutant virions were approximately eightfold lower for the PB1 and M genes and about fivefold lower for than HA gene than were those of the rg-B/Y wt virions (Fig. 7C). This result indicated that the decrease in NP and M1 is the result of inhibited incorporation of the vRNP complex into the virion. Taken together, the data suggest that BM2 is responsible for the incorporation of the vRNP complex into virion.

Truncated BM2 affects incorporation of vRNP into virions. It was previously shown that the truncated BM2 (deletion from amino acids [aa] 51 to 80) was transported to the plasma membrane as normally seen in wt BM2 (32). The BM2Δ51–80 mutant virus, however, was not recovered by the reverse ge-

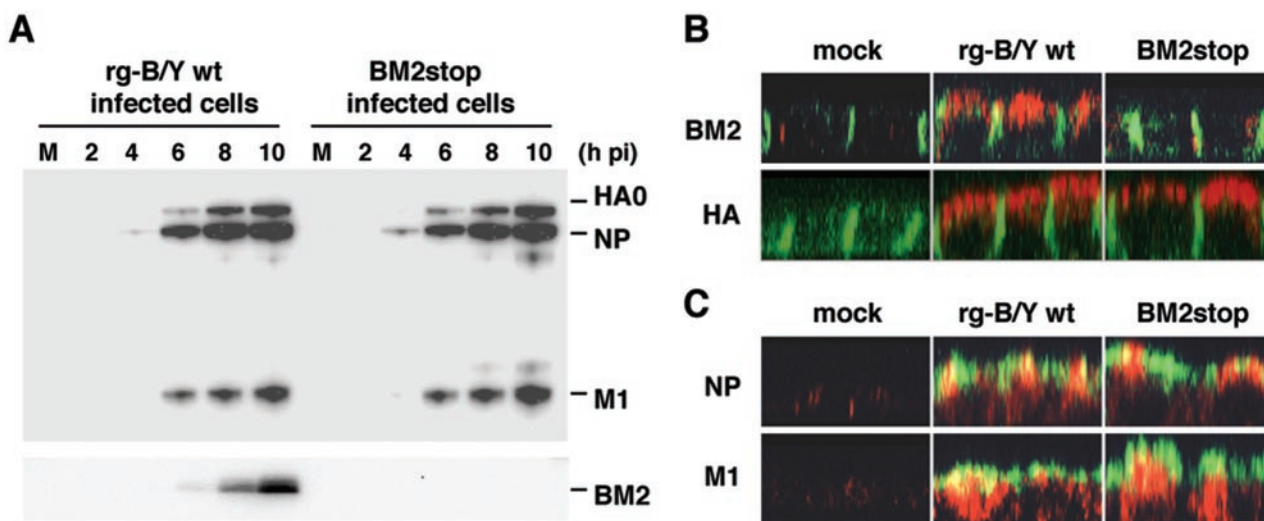


FIG. 6. Synthesis and localization of viral proteins in virus-infected MDCK cells. (A) Time course of viral protein synthesis of BM2stop mutant and rg-B/Y wt viruses. Viral proteins were detected by Western blotting with either anti-B/Yamagata (upper panel) or anti-BM2 (lower panel) antibody. Lane M is the lysate of uninfected cells. (B and C) Intracellular localization of the viral proteins of BM2stop mutant and rg-B/Y wt viruses. At 11 hpi, infected cells were fixed and subjected to IFA. Panels show confocal microscopy images of XZ sections, with the apical membrane uppermost. (B) BM2 (red) and HA (red) were detected together with β -catenin (green). β -Catenin is located at the lateral membrane. (C) NP (red) and M1 (red) were detected together with HA (green).

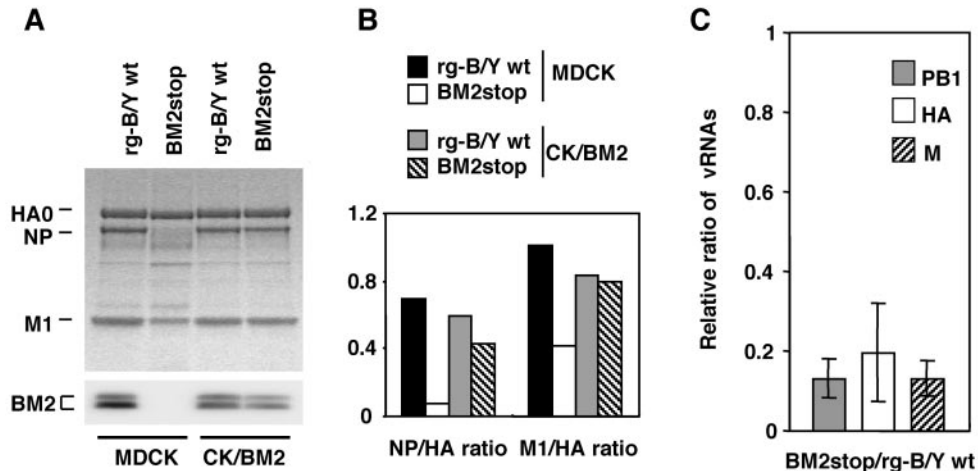


FIG. 7. Viral proteins and RNA segments in BM2stop mutant virus particles. (A) Protein composition of BM2stop mutant and rg-B/Y wt virions. The purified virions that were produced by MDCK and CK/BM2 cells were analyzed by Coomassie brilliant blue staining (upper panel) and Western blotting with anti-BM2 antibody (lower panel). (B) Relative amounts of viral proteins. Viral proteins were quantified by using an ATTO CS analyzer, and the relative staining intensity of each protein was normalized to that of HA (set at 1.00) for each virus. (C) Relative ratios of PB1, HA, and M genes between BM2stop and rg-B/Y wt virions. RNA was extracted from the virions grown in MDCK cells and reverse transcribed, and the resultant cDNA was quantitated by real-time PCR with primers directed to the PB1, HA, and M genes (see Materials and Methods). The mutant virions analyzed had amounts of HA proteins equal to those of rg-B/Y wt. All samples were run in duplicate and repeated three times. Error bars represent the standard errors of the means.

netics system (Table 1) but was recovered when wt BM2 was supplemented from CK/BM2 cells (data not shown). The recovered mutant virus synthesized a truncated BM2 in infected MDCK cells (Fig. 8A), and this product was found at the apical membrane, similar to that of wt BM2, in an XZ section by IFA (Fig. 8B).

The components of the purified virion of BM2 Δ 51–80 mutant virus were also examined in parallel with that of rg-B/Y wt virus (Fig. 8C). In the virions produced by MDCK cells, dramatic reductions in NP and M1 were observed, although the truncated BM2 was incorporated into the virions. In contrast, in the virions produced by CK/BM2 cells, the amounts of NP and M1 were approximately 70 and 90%, respectively, of the levels seen in rg-B/Y wt virus. These observations were consistent with the findings for BM2stop mutant virus, indicating that the loss of intrinsic BM2 expression and the loss of BM2 itself resulted in the suppression of vRNP incorporation into virions. Interestingly, the BM2 Δ 51–80 mutant virus grown in CK/BM2 cells contained two species of BM2, the wt BM2 supplied from CK/BM2 host cells and the truncated BM2, in different amounts. The levels of incorporation of these proteins were reflected by the levels of their syntheses in infected cells.

Membrane association of M1 is greatly suppressed in the absence of BM2. During the assembly process, viral components are transported to the budding site of the plasma membrane and interact directly or indirectly with the membrane (2). To clarify why incorporation of vRNP into virions was greatly affected in the absence of BM2, the membrane association of the viral proteins was examined by sucrose flotation centrifugation analysis. Figure 9 shows the fractions of infected cell lysates collected at 11 hpi followed by Western blotting with anti-B/Yamagata and anti-BM2 antibodies. Fractions 8 and 9 were found to mainly contain cellular membranes in a

previous study (32). In MDCK cells, at least half of the total amount of HA and M1 of rg-B/Y wt virus was detected in the membrane fractions and part of NP was also found in fraction 8 (Fig. 9A, top panel). In contrast, the majority of the M1 in the BM2stop and BM2 Δ 51–80 mutant viruses was found in cytosolic fractions 1 to 4 and only a trace amount of M1 was detected in the membrane fractions, although the membrane-associated HA did not greatly differ from that in rg-B/Y wt virus (Fig. 9B and C, top panels). The substantial reduction in membrane-associated M1 may correspond to the decrease in NP, probably vRNP, in the membrane fractions. On the other hand, with *trans*-supplementation of BM2 from CK/BM2 host cells, no suppression of the membrane association of M1 was observed (Fig. 9B and C, bottom panels). Because the distribution of M1 from BM2stop mutant viruses at the apical membrane of MDCK cells did not differ from that of rg-B/Y wt virus (Fig. 6C), these results indicate that the membrane association of M1 was highly affected in the absence of BM2 on the apical membrane, resulting in the failure of packaging of the vRNP complex into the virions.

DISCUSSION

BM2 is an integral membrane protein that is transported to the plasma membrane (28, 32) and is incorporated into virions as a minor component (27). Electrophysiological studies of BM2 in *Xenopus* oocytes and in mammalian cells demonstrated that BM2 has proton (H⁺) ion channel activity as a functional counterpart of the influenza A virus M2 protein (24). To further explore the role of BM2 in the life cycle of influenza B virus, we generated knockout and truncated mutant viruses of BM2 by using a newly developed reverse genetics system for influenza B virus.

The BM2 Δ ATG mutant virus, in which the initiation codon

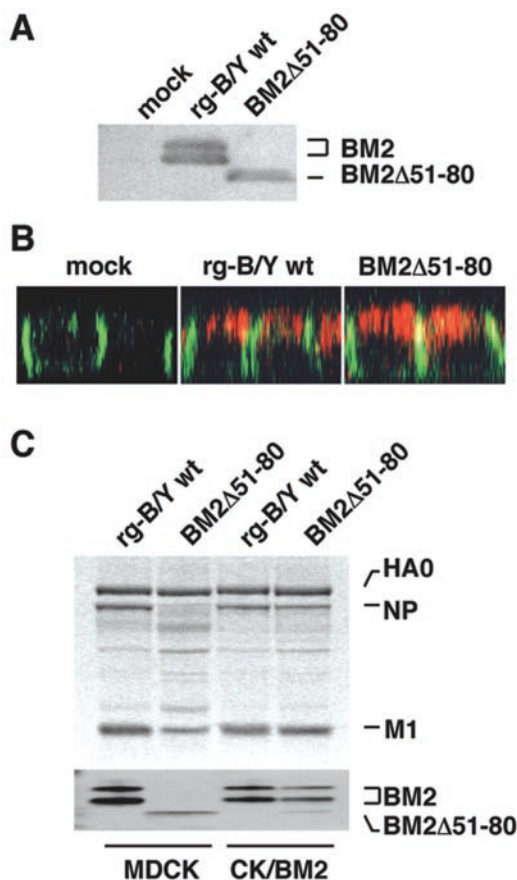


FIG. 8. Characterizations of viral proteins of BM2Δ51-80 mutant viruses in infected cells and purified virions. (A) BM2 proteins in MDCK cells were analyzed by Western blotting with anti-BM2 antibody. (B) The localization of BM2 proteins at the apical membrane of MDCK cells was examined by IFA with anti-BM2 antibody (BM2 is shown in red) together with anti-β-catenin antibody (β-catenin is shown in green). (C) The protein composition of the mutant and rg-B/Y wt virions was analyzed as described for Fig. 7.

for BM2 translation ($_{771}$ AUG) was replaced with $_{771}$ ACC, was recovered at levels similar to those of the wt virus from plasmid-transfected cells (Table 1). This mutant virus grew more slowly than wt virus in the multiple-step growth cycle but produced progeny viruses with a maximum yield similar to that of wt virus (Fig. 2). Although the BM2 ORF of the mutant virus did not contain an initiation codon, BM2 was synthesized at low levels in infected cells and was detected in the virions (Fig. 2). BM2 and influenza A virus M2 have been shown to have similar ion channel activities (24). The ion channel of influenza A virus M2 is thought to be important for dissociation of M1 from RNP by weakening the protein-protein interactions by acidification from pH 7.4 to 5.8 during uncoating (5, 6, 22, 39, 41), resulting in the subsequent transport of vRNP into the nucleus, where replication and transcription of the virus genome occur (20). If BM2 ion channel activity is crucial, it is possible that inefficient uncoating as a result of reduced expression of the ion channel protein BM2 is one of the reasons for the slow growth of the BM2ΔATG mutant virus. However, the final virus yield of the mutant virus was similar to that of

the wt virus, indicating that a minimum amount of BM2 can support full growth of influenza B virus.

The question of how BM2 is synthesized without the AUG initiation codon in the ORF thus arises. The synthesized BM2 had similar molecular mass, 12 kDa (15, 27), to wt BM2. In the +2 ORF of RNA segment 7, in which BM2 is translated (15), there are three AUG triplets, $_{831}$ AUG, $_{1002}$ AUG, and $_{1062}$ AUG, which are potential initiation codons downstream of the $_{771}$ AUG BM2 initiation codon, but there are none upstream. If translation occurred from any of these triplets, the products would lack the transmembrane domain (TMD), which is translated from $_{771}$ AUG to $_{837}$ TGG, like the BM2Δ2-23 mutant (32). The mutant lacking the TMD in the BM2 molecule was not recovered at all, as shown in Table 1, because the BM2 molecule without TMD is not properly transported to the budding site on the plasma membrane (32). Moreover, the putative molecular masses of the products would be 10, 3.6, or 1.4 kDa. These products are smaller than wt BM2, and if such small products are produced, they could be distinguished from wt BM2, indicating that these triplets are not used for BM2 synthesis in the BM2ΔATG mutant virus. Moreover, BM2 of the mutant virus reacted with an anti-BM2 antibody against wt BM2 protein and functioned as wt BM2 to produce infectious viruses, thus suggesting that the amino acid alignment between the BM2ΔATG mutant and wt viruses is the same. It is therefore most likely that the BM2 of the BM2ΔATG mutant virus is translated from the $_{771}$ ACC replacement codon. Similar unusual translation initiated from GUG or UUG triplets is observed with *Escherichia coli*, although their translational efficiency is considerably lower than that initiated from the AUG triplet (19).

By inserting three consecutive stop codons downstream of the $_{771}$ ACC replacement codon in BM2ΔATG plasmid DNA, a knockout virus of BM2 was successfully generated in a BM2 trans-supplementation system. This BM2 knockout virus permitted production of infectious viruses only when wt BM2 was supplemented by host cells expressing BM2 (Fig. 5). Consequently, it was apparent that BM2 is a necessary component for the production of infectious progeny viruses. Mould et al. (24) recently indicated that overexpression of BM2 causes morphological changes in the Golgi apparatus and a delay in exocytic transport of HA when BM2 and HA of influenza A/FPV (Rostock strain) virus were coexpressed in HeLa cells. However, we did not observe this disadvantageous virus growth because the growth of wt virus in the CK/BM2 cell line, in which BM2 was expressed by host cells in addition to the expression by virus itself, did not differ from that in MDCK cells (Fig. 5). Presumably, the expression level of BM2 does not influence the normal growth of influenza B virus in tissue culture.

Previously, Watanabe et al. (33) suggested that the ion channel activity of influenza A virus is dispensable for growth in tissue culture, whereas Takeda et al. (30) subsequently argued their findings by analysis of amantadine sensitivity and concluded that ion channel activity contributes to the efficient replication of virus. Although the ion channel activity of BM2 may be important for the growth of influenza B virus, our study of BM2 knockout virus suggested another function of BM2 in the life cycle of influenza B virus. In the absence of BM2 expression, the synthesis and cytoplasmic transport of major

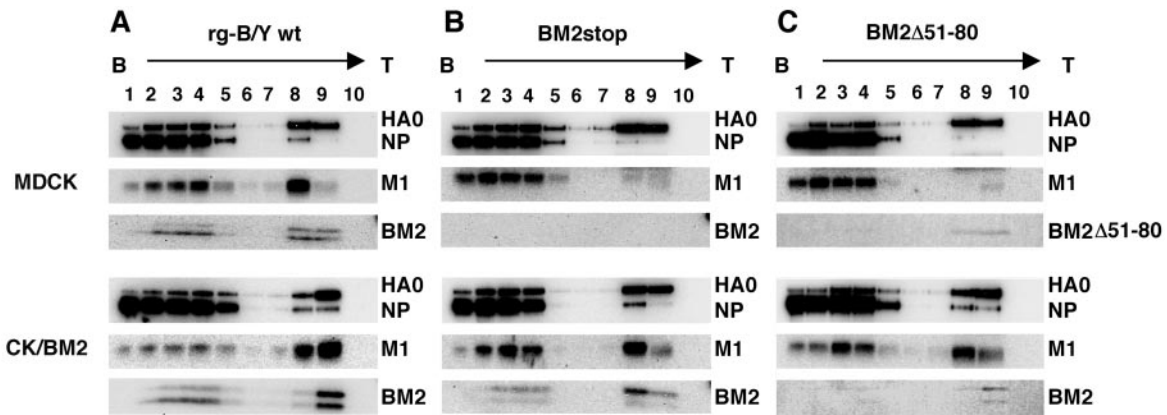


FIG. 9. Membrane association of viral proteins in virus-infected cells. MDCK and CK/BM2 cells were infected with rg-B/Y wt (A), BM2stop (B), and BM2 Δ 51–80 (C) viruses. At 11 hpi, infected cells were lysed and postnuclear fractions were subjected to equilibrium centrifugation as described in Materials and Methods. Viral proteins in all fractions collected from the top (T) to the bottom (B) were analyzed by Western blotting with anti-B/Yamagata and anti-BM2 antibodies.

viral proteins, HA, NP, and M1, to the virion budding site occurred normally and were indistinguishable between the BM2 knockout and wt viruses (Fig. 6). The most striking difference was found in the content of the vRNP complex, NP and M1, in virions when comparing viruses produced in the absence and presence of BM2 (Fig. 7A). These differences were also found in the case of loss of intrinsic BM2 expression by internal deletion of the molecule, even when the truncated BM2 was synthesized and integrated into the plasma membrane, as occurs in the wt virus (Fig. 8C). Therefore, it is most likely that BM2 plays a role in the incorporation of the vRNP complex into the virion during the assembly process. This interpretation does not conflict with the observation that suppressed synthesis of wt BM2 correlates to a decrease in the content of vRNP, as calculated by the NP/HA ratio in the BM2 Δ ATG mutant virion (Fig. 2).

In influenza A viruses, M1 has lipid-binding properties (11, 29). The cytoplasmic tails of HA and NA glycoproteins on the plasma membrane are known to stimulate the membrane association of M1 to form a shell lining the inner surface of the viral envelope (7, 10). Unlike influenza A virus, the assembly mechanism of virus components at the virion budding site on the plasma membrane are not well characterized for influenza B virus. In the present study, we confirmed by XZ section observation of infected cells by confocal microscopy that influenza B virus M1 clearly underlined the apical membrane containing HA (Fig. 6). If the cytoplasmic tails of the envelope proteins HA, NA, and NB mediate the membrane association of M1, as in the influenza A virus, their interactions would not be so close. Our results with BM2 knockout virus indicated that the membrane association of influenza B virus M1 was highly influenced by the presence and the absence of BM2 on the membrane. In fact, flotation gradient centrifugation analysis revealed that the membrane association of M1 was greatly affected by the absence of BM2, whereas this decreased membrane association of M1 was restored when wt BM2 was supplemented *in trans* by host cells expressing BM2 (Fig. 9). Based on these results, it is likely that BM2 stimulates the binding of M1, presumably through an M1-BM2 interaction, to the apical membranes. This M1-BM2 interaction was suggested by our

previous experiments in which BM2 was coprecipitated with M1 when detergent-disrupted virion was immunoprecipitated with an anti-M1 antibody, and in experiments with virion fractionation, BM2 always coexisted with M1 in the same fractions but was not present with HA and vRNP without M1 (27). Moreover, the longest cytoplasmic tail of BM2 may contribute to the capture of M1 at the membrane site because the cytoplasmic tails of BM2, HA, NA, and NB are deduced to be 84 to 86 aa residues (28, 32), 10 aa residues (8), 12 aa residues (8), and 60 aa residues (35), respectively. This interpretation is consistent with the result that the shortened cytoplasmic tail of BM2 Δ 51–80 mutant virus, which possesses a 30-aa deletion in the BM2 molecule, resulted in a failure to stimulate the membrane association of M1, similar to the BM2 knockout virus after flotation centrifugation (Fig. 9C). Taken together, these findings suggest that BM2 interacts with M1 at the plasma membrane and that this BM2-M1 interaction may be necessary for the tight association of M1 to the plasma membrane.

In the case of influenza A virus, M1 interacts with vRNP through direct binding with vRNA and interaction with NP (31, 36), and this complex formation is thought to be necessary to capture the vRNP complex at the virion budding site during the assembly process. Similarly, in the case of influenza B virus, vRNP was also suggested to form a complex with M1 by our virion fractionation experiments (16). This complex formation would also be necessary to capture vRNP at the virion budding site like influenza A virus. Consequently, the significant decrease in membrane binding of M1 in the absence of BM2 may result in reduced incorporation of the M1-vRNP complex into virions.

In the particles of the BM2stop mutant and BM2 Δ 51–80 mutant viruses, however, M1 was still detectable, although the content of M1 was greatly decreased together with the lack of vRNP incorporation into virion (Fig. 7 and 8). The results may imply that there are two forms of M1 in the virion, i.e., M1 with vRNP and M1 without vRNP. The lack of vRNP with the decrease of M1 in the virion in the absence of BM2 suggests that BM2 plays an important role in the tight membrane association and subsequent incorporation of the M1-vRNP complex during the virion assembly process. The precise function

of BM2 on vRNP packaging into virions should be clarified to elucidate the mechanism of influenza B virus assembly.

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