Influenza B Virus BM2 Protein Is Transported through the trans-Golgi Network as an Integral Membrane Protein

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A bicistronic mRNA transcribed from the influenza B virus RNA segment 7 encodes two viral proteins, matrix protein M1 and uncharacterized small protein BM2. In the present study, we focused on the cytoplasmic transport and cellular membrane association of BM2. Immunofluorescence studies of virus-infected cells indicated that BM2 accumulated at the Golgi apparatus immediately after synthesis and then was transported to the plasma membrane through the trans-Golgi network. Localization of a set of BM2 deletion mutants revealed that the N-terminal half of BM2 (residues 2 to 50) was crucial for its transport; in particular, the deletion of residues 2 to 23, deduced to be a transmembrane domain, resulted in diffused distribution of the protein throughout the entire cell. Sucrose gradient flotation and biochemical analyses of the membrane showed that BM2 was tightly associated with cellular membranes as an integral membrane protein. Oligomerization of BM2 was demonstrated by coprecipitation of differentially epitope-tagged BM2 proteins. Taken together, these results strongly suggest that BM2 is integrated into the plasma membrane at the N-terminal hydrophobic domain as fourth membrane protein, in addition to hemagglutinin, neuraminidase, and NB, of the influenza B virus.

The influenza A, B, and C viruses are segmented RNA viruses of negative polarity and assemble at and bud from the plasma membrane of virus-infected cells. Influenza A and B viruses are structurally and biochemically similar to each other. The envelopes of influenza A and B viruses contain three kinds of integral membrane proteins: two major glycoproteins, hemagglutinin (HA) and neuraminidase, and a small protein, either M2 in influenza A virus or NB in influenza B virus (19). The proteins are functionally analogous to each other in the life cycle of each virus. HA plays two major roles during viral replication: attachment of the virus to the cell surface and fusion of the host cell membranes and viral envelope, leading to viral penetration (19). Neuraminidase acts as a receptor-destroying enzyme to promote the release of progeny viruses from the host cells and to prevent virion aggregation (22, 29). M2 of influenza A virus acts as an ion channel to permit protons to enter the virions during uncoating of particles in the endosomes (12, 23, 34). This ion channel activity also equilibrates pH between the lumen of the trans-Golgi network (TGN) and the cytoplasm, thereby inhibiting expression of the membrane fusion activity of HA by preventing conformational changes in HA (e.g., A/fowl plague/Rostock/34) at low pH in this compartment (11, 28, 36). NB of the influenza B virus also has ion channel activity detected by Escherichia coli-expressed NB in planar bilayers (35) and is thought to be the functional counterpart of M2 (20). These small integral membrane proteins, M2 and NB, lack a cleavable signal sequence and have a single transmembrane domain, which consists of 19 and 22 hydrophobic or uncharged amino acid residues, respectively (21, 38, 39). Furthermore, these proteins are integrated in the N-terminal extracellular and the C-terminal intracellular orientations (21, 38, 39).

In addition to the proteins mentioned above, influenza B virus produces a unique protein, BM2, whose features and functions in the life cycle of the virus are still unclear. The BM2 protein is encoded by bicistronic mRNA derived from RNA segment 7 of influenza B virus, which also encodes the matrix protein M1 (5, 13). The translational strategy of BM2 is unique. The initiation codon of BM2 overlaps the termination codon of M1 in an overlapping stop-start pentanucleotide, TAATG, at nucleotides 769 to 773 (16). BM2 synthesis is triggered by the termination of translation of M1, which is encoded in the upstream region of mRNA (16). Since the amino acid sequence of BM2 has been highly conserved for at least 59 years, from B/Lee/40 (5) to B/Nagoya/20/99 (Influenza Sequence Database, http://www.ﬂu.lanl.gov) and since BM2 is detected in cells infected with all influenza B viruses examined so far (16, 26), BM2 is thought to play an important role(s) in the life cycle of influenza B virus. We have previously shown with virus-infected cells that BM2 is a phosphoprotein synthesized in the late phase of infection and is localized in the perinuclear region, probably at the Golgi apparatus, immediately after its synthesis. Thereafter, BM2 is transported to the plasma membrane, resulting in its incorporation into the virion (26). Although transport of BM2 from the perinuclear region to the plasma membrane seems similar to that of other mem-
Our results indicate that BM2 is an integral membrane protein transported to the plasma membrane through the TGN and that BM2 forms an oligomer. Some of these findings were consistent with a recent report published while this work was in progress (30).

**BM2 accumulates at the Golgi apparatus after synthesis and is transported to the plasma membrane through the TGN in infected cells.** We have previously demonstrated that BM2 accumulates in the perinuclear region after synthesis in infected cells (26). Since this region was expected to be the Golgi apparatus, we explored whether BM2 colocalized with the Golgi apparatus of virus-infected cells. To this end, MDCK cells were transfected with a plasmid (pEYFP-Golgi; Clontech) expressing the yellow fluorescent protein (YFP) for specific labeling of the trans-medial region of the Golgi apparatus. The cells were then infected with B/Yamagata/1/73 virus. By immunofluorescence assay (IFA), the Golgi apparatus was observed at the perinuclear region in MDCK cells (Fig. 1A, left panel). The same cells were fixed with 10% phosphate-buffered formalin and permeabilized with 0.05% Triton X-100 in phosphate-buffered saline and then incubated with rabbit anti-BM2 antibody (26) to detect BM2 at 4 h postinfection. BM2 was detected at the perinuclear region (Fig. 1A, middle panel), in agreement with our previous observation, and its location completely overlapped that of the Golgi apparatus (Fig. 1A, right panel). In mock-infected cells, YFP was detected at the Golgi apparatus, but BM2 was not detected at all (data not shown). The localization of BM2 and the endoplasmic reticulum (ER) was also examined by IFA. We did not, however, observe precise colocalization of BM2 and the ER (data not shown). These results indicate that BM2 accumulates at the Golgi apparatus immediately after synthesis in infected cells.

We have also previously demonstrated that BM2 is transported from the perinuclear region to the plasma membrane with increasing incubation time (26). It is likely that BM2 is transported to the plasma membrane through the TGN, as has been demonstrated with the membrane glycoprotein HA (9, 32). To examine whether the cytoplasmic transport of BM2 is similar to that of HA, the localization of BM2 and HA over time were detected in virus-infected cells by indirect IFA using rabbit anti-BM2 and mouse anti-HA antibodies. As shown in Fig. 1B, both HA and BM2 were detected at the Golgi apparatus to the plasma membrane throughout infection and their cellular locations overlapped (Fig. 1B, right panels), indicating that BM2 is transported to the plasma membrane by the same pathway as HA. We next treated infected cells with monensin, which is known to delay or block the transport of many integral membrane proteins from the medial region of the Golgi apparatus to the plasma membrane (2, 10). At 2 h postinfection (p.i.), the virus-infected cells were treated with or without 10 μM monensin (final concentration) and then incubated further for 8 h at 34°C. In the absence of monensin, both HA and BM2 predominantly stayed around the Golgi apparatus (Fig. 1C, lower panels). These results suggest that BM2, like HA, is transported to the plasma membrane through the TGN. Furthermore, these findings were confirmed for cells transfected with a plasmid expressing BM2, indicating that BM2 transport to the plasma membrane through the TGN occurs independently of HA and other viral proteins (data not shown).

**The N-terminal region of BM2 is important for its cytoplasmic transport.** We next investigated which domain(s) of BM2 is important for its cytoplasmic transport. To this end, deletion mutants of BM2 were generated as shown in Fig. 2A. The mutant genes were cloned into the mammalian expression plasmid pCAGGS/MCS (24, 37) and were sequenced to ensure that unwanted mutations were not present. These constructs were transfected into human embryonic kidney 293T cells, and the predicted sizes of the resulting proteins were analyzed by Western blotting with anti-BM2 antibody. Although a plasmid expressing the mutant BM2Δ78-109 (deletion of amino acids 78 to 109) was constructed, this mutant protein was not detected with anti-BM2 antibody. The antibody may recognize mainly the C terminus of the BM2 molecule. Thus, we did not analyze this construct further in the present study. The BM2 protein of most influenza B viruses is a single band, while two forms of BM2 (less phosphorylated and phosphorylated) are detected in B/Yamagata/1/73 (26). As shown in Fig. 2B, we detected two bands (a major and a minor band) in wild-type (wt) BM2 and the mutants. We also detected not only wt BM2 but also both mutants BM2Δ2-23 (deletion of amino acids 2 to 23) and BM2Δ24-50 (deletion of amino acids 24 to 50) at reasonable molecular sizes. Mutant BM2Δ51-80 (deletion of amino acids 51 to 80), however, was larger than the expected size. The difference in its electrophoretic mobility may reflect a difference in amino acid composition. In addition, it is noteworthy that we could see larger bands than expected in wt and mutant BM2Δ2-23 (D and T in the wt and D in BM2Δ2-23). Since we did not detect any bands in mock-transfected cells (data not shown), these larger bands were deduced to be an oligomer (see below).

To identify the intracellular localization of the BM2 mutants, plasmids expressing BM2 mutants were transfected into MDCK cells and the cytoplasmic transport of these proteins was analyzed by IFA. The mutant BM2Δ51-80 was detected at the Golgi apparatus at 8 h posttransfection (p.t.) (Fig. 2C, panel b) and even at the plasma membrane at 24 h p.t. (Fig. 2C, panel f), indicating that this mutant protein as well as wt BM2 was transported to the plasma membrane (Fig. 2C, panels a and e). The mutant BM2Δ24-50 was found at the Golgi apparatus at 8 h p.t. (Fig. 2C, panel c), but a large part of this protein remained at the Golgi apparatus at 24 h p.t. (Fig. 2C, panel g). Interestingly, the localization of BM2Δ2-23 was quite different from that of wt BM2 and the other mutants. This mutant localized at neither the Golgi apparatus nor the plasma membrane, but it diffused throughout the entire cell, including the nucleus, during its expression (Fig. 2C, panels d and h). These results suggest that amino acids 2 to 50 at the N terminus are important for BM2 transport through the TGN.

**BM2 associates with cellular membranes as an integral...**
FIG. 1. (A) Accumulation of BM2 at the Golgi apparatus in B/Yamagata/1/73 virus-infected cells. Plasmid pEYFP-Golgi, which expresses YFP that targets the trans-medial region of the Golgi apparatus, was transfected into MDCK cells, and at 24 h p.t., B/Yamagata/1/73 virus was used to infect the cells. At 4 h p.i., cells were fixed and BM2 was detected by IFA using anti-BM2 antibody. Images of the Golgi apparatus and BM2 were merged. (B) Distribution of HA and BM2 in virus-infected cells. Virus-infected cells were incubated for 4, 8, and 10 h. At the indicated times, cells were fixed and HA (left panels) and BM2 (middle panels) were detected by IFA using mouse anti-HA and rabbit anti-BM2 antibodies, respectively. Both images were merged (right panels). (C) Effects of monensin treatment on the distribution of HA and BM2 in virus-infected cells. Virus-infected cells were treated without (upper panels) or with (lower panels) monensin at 2 h p.i. and were further incubated for 8 h. After fixation of cells, HA (left panels) and BM2 (middle panels) were detected by IFA using mouse anti-HA and rabbit anti-BM2 antibodies, respectively. Both images were merged (right panels).
membrane protein. As shown above by IFA, BM2 was expected to associate with cellular membranes. Thus, the membrane association of BM2 was investigated by separating the cellular membrane fraction from the cytosolic proteins of cells transfected with a plasmid expressing BM2 by sucrose flotation centrifugation (1, 7). All fractions were analyzed by Western blotting with anti-B/Yamagata/1/73 (26) and anti-BM2 antibodies. HA, a membrane protein, or NP, a cytosolic protein, was also independently expressed in MDCK cells, and their membrane associations were analyzed in parallel with that of BM2. HA was detected at the top of the gradient, whereas NP remained at the bottom of the gradient (Fig. 3A, top and middle panels), indicating that cellular membranes are well separated from cytosolic materials by this procedure. The membrane fractions were also confirmed by detection of Na⁺K⁺-ATPase as a positive control (data not shown). Under this condition, BM2 was detected at the top of the gradient (Fig. 3A, bottom panel). The result indicates that BM2 can associate with cellular membranes and that other viral proteins are not required for this association.

Computer analysis of the amino acid sequences of BM2 among many influenza B viruses indicated that the N terminus was highly conserved and highly hydrophobic (data not shown), implying that this region functions as a transmembrane domain. To examine whether BM2 is integrated into, not just attached to, cellular membranes, the membrane fractions containing BM2 were treated with 2 M KCl, 50 mM EDTA, or carbonate buffer (pH 10) and then subjected to a second round of sucrose flotation centrifugation. These treatments specifically remove the peripheral proteins associated on membranes but not integral membrane proteins (6, 8). As shown in Fig. 3B, these treatments failed to dissociate BM2 from the cellular membranes. To further characterize the association of BM2 with cellular membranes, we conducted Triton X-114 phase-partitioning analysis on membrane fractions containing either BM2 or HA. In this assay, integral membrane proteins or lipid-anchored proteins are partitioned to the detergent phase, whereas peripheral membrane proteins are partitioned to the aqueous phase (3). As shown in Fig. 3C, HA, which is known to be an integral membrane protein, was extracted in the detergent phase as expected. BM2 was partitioned to the detergent phase as well. Taken together, these results strongly suggest that BM2 is an integral membrane protein. The finding is consistent with that reported by Paterson et al. (30).

The BM2 protein forms an oligomer. As mentioned earlier (Fig. 2B), it seems that BM2 can form an oligomer. To verify this, we generated plasmids expressing differentially epitope-tagged BM2. Either the FLAG or His epitope was added to the C terminus of BM2 (BM2FLAG and BM2His, respectively). Plasmids expressing BM2FLAG or BM2His were transfected into 293T cells separately or together. The expression of these tagged BM2 proteins was confirmed by Western blotting with anti-BM2 antibody (Fig. 4A). These fusion proteins were also specifically detected by anti-FLAG and anti-His antibodies, without cross-reactivity (Figs. 4B and C). Immunoprecipitation (IP) of the cell lysates with rabbit anti-FLAG antibody (Sigma) followed by Western blotting with mouse anti-His6 antibody (Roche) showed that BM2His was detected only in cells where it was expressed with BM2FLAG (Fig. 4B). Similar results were obtained by IP with anti-His6 antibody, followed by Western blotting with anti-FLAG antibody (Fig. 4C). To exclude the possibility that BM2 detected after IP was an aggregate, the
lysates of cells separately expressing BM2FLAG or BM2His were mixed and subjected to IP with anti-FLAG or anti-His$_6$ antibody, followed by Western blotting with anti-His$_6$ or anti-FLAG antibody, respectively. In this case, no specific band of BM2 was detected (Fig. 4B and C, lane Mix). Consequently, these results indicate that both tagged BM2 proteins associated with each other in host cells, suggesting that BM2 forms an oligomer.

We have previously demonstrated that BM2 is a phosphoprotein synthesized in the late phase of infection and is localized in the cytoplasm throughout infection and that BM2 is transported from the perinuclear region to the plasma membr-
brane and thereafter is incorporated into virions (26). In the present study, we further clarified that BM2 was colocalized with the Golgi apparatus after synthesis and that the cytoplasmic transport of BM2 was mirrored by that of HA (Fig. 1), which is well characterized as a membrane protein transported by the TGN pathway (9, 32). Results suggest that BM2 is transported to the plasma membrane through the TGN. This finding is also supported by the result that BM2 transport from the Golgi apparatus to the plasma membrane was greatly suppressed by monensin treatment (Fig. 1), which delays or blocks the exocytic pathway of integral membrane proteins from the medial region of the Golgi apparatus to the plasma membrane (2, 10). In addition, BM2 expressed alone in the absence of other viral proteins revealed similar localizations in virus-infected cells (data not shown), suggesting that the cytoplasmic transport of BM2 to the plasma membrane through the TGN is its intrinsic character.

The localization of deletion mutants of BM2 in transfected cells revealed regions important for normal cytoplasmic transport (Fig. 2). The mutant BM2Δ51-80 exhibited a distribution similar to that of wt BM2, indicating that amino acid residues 51 to 80 of BM2 were not required for normal transport. In contrast, two mutants, BM2Δ24-50 and BM2Δ2-23, showed remarkably different localizations from that of wt BM2. The mutant BM2Δ24-50 remained at the Golgi apparatus and was not transported to the plasma membrane. This restricted localization was quite similar to that of wt BM2 in monensin-treated cells (Fig. 1). The result may imply that mutant BM2Δ24-50 may lose the ability to interact with the host components necessary for transport from the Golgi apparatus or that the mutant BM2Δ24-50 may gain an unidentified Golgi retention signal by the deletion of amino acid residues 24 to 50, although the known retention motifs reported by others (4, 25) were not found in BM2Δ24-50 proteins. On the other hand, the mutant BM2Δ2-23 diffused throughout the entire cell, including the nucleus. Analysis of the predicted amino acid sequence indicates that BM2 likely contains a single hydrophobic domain at the N terminus, which would be sufficient to be integrated into membranes (data not shown). Biochemical treatments, however, failed to dissociate BM2 from the cellular membranes (Fig. 2). Moreover, a Triton X-114 phase-partitioning assay revealed that BM2, like HA, was partitioned to the detergent phase (Fig. 3). These results, together with the results of the IFA of mutant BM2Δ2-23, strongly suggest that a hydrophobic domain at the N terminus of BM2 interacts integrally with cell membranes. We did not experimentally exclude the possibility that this hydrophobic region might be a cleavable signal peptide. If this is the case, however, the BM2 molecule would need another hydrophobic region for integration into the membrane. Thus, this region is likely to be a transmembrane domain, not a cleavable signal peptide, although we did not assess whether or not BM2 mutants can associate with membranes.

The localization of BM2 and the ER was also examined by IFA. We did not observe the precise localization of BM2 at the ER (data not shown). This may suggest that the anti-BM2 antibody used in the present study did not recognize the epitope on the BM2 molecule during its synthesis at the ER or that the transport of BM2 from the ER to the Golgi apparatus occurred rapidly. It remains also to be determined how BM2 initially interacts with membranes during its synthesis at the ER, whether by cotranslational translocation (signal recognition particle [SRP] dependent) or posttranslational translocation (SRP independent). M2 of the influenza A virus contains a single noncleavable internal hydrophobic domain, which acts both as an SRP-dependent signal sequence and a stop-transfer sequence to be the transmembrane domain (17). As discussed above, BM2 also contains a single hydrophobic domain and has been suggested to have an N terminus-out–C terminus-in
orientation in membranes, as M2 does (21, 30, 39). Thus, BM2, like M2, is probably integrated into membranes by an SRP-dependent mechanism, and the hydrophobic domain presumably acts as a stop-transfer sequence for the integration.

The small integral membrane proteins M2 and NB have been shown to form disulfide-linked dimers and tetramers (15, 34, 38). Analysis of the coimmunoprecipitation of the differentially tagged BM2 proteins, BM2FLAG and BM2His, also strongly suggests that BM2 can form an oligomer (Fig. 4). Unlike M2 and NB proteins, however, the single-cysteine residue in BM2 is at position 11 within the N-terminal hydrophobic domain, which is most likely located inside the membrane. This finding suggests that BM2 cannot form an oligomer by disulfide-linked interaction. Indeed, we could detect larger forms of BM2 under reducing conditions (Fig. 2B). M2 and NB have an ion channel activity, produced by generating a pore through oligomer formation (31, 33–35). Since BM2 also likely generates a pore in the membrane by forming an oligomer, BM2 may have an ion channel activity as well. This issue remains to be investigated.

What are the functions of BM2 in the viral life cycle? Using immunoprecipitation assays, fractionation of viral ribonucleoprotein (vRNP) complexes, and the two-hybrid system (27), members of our laboratory have previously demonstrated that BM2 can interact, through encapsidated M1 and probably NS2, with vRNP complexes. We demonstrated here that BM2 alone could be transported to the plasma membrane. Taken together, our results indicate that BM2 can probably associate with vRNP complexes at the budding site, suggesting that BM2 may play an important role(s) in virus assembly and budding. Recently, a reverse genetics system for influenza B virus was established (14, 18; S. Watanabe, M. Imai, and T. Odagiri, unpublished data). This system is a potential tool for determining the functions and importance of BM2 in the viral life cycle.

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