Developmental Cell, Vol. 5, 175–184, July, 2003, Copyright ©2003 by Cell Press

Influenza B Virus BM2 Protein Has Ion Channel Activity that Conducts Protons across Membranes

Jorgen A. Mould,[1,4] Reay G. Paterson,[1,4] Makoto Takeda,[1,2] Yuki Ohigashi,[1] Padma Venkataraman,[3] Robert A. Lamb,[1,2,*] and Lawrence H. Pinto[3]

[1]Department of Biochemistry, Molecular Biology and Cell Biology

[2]Howard Hughes Medical Institute

[3]Department of Neurobiology and Physiology

Northwestern University

Evanston, Illinois 60208

Summary

Successful uncoating of the influenza B virus in endosomes is predicted to require acidification of the interior of the virus particle. We report that a virion component, the BM2 integral membrane protein, when expressed in *Xenopus* oocytes or in mammalian cells, causes acidification of the cells and possesses ion channel activity consistent with proton conduction. Furthermore, coexpression of BM2 with hemagglutinin (HA) glycoprotein prevents HA from adopting its low-pH-induced conformation during transport to the cell surface, and overexpression of BM2 causes a delay in intracellular transport in the exocytic pathway and causes morphological changes in the Golgi. These data are consistent with BM2 equilibrating the pH gradient between the Golgi and the cytoplasm. The transmembrane domain of BM2 protein and the influenza A virus A/M2 ion channel protein both contain the motif HXXXW, and, for both proteins, the His and Trp residues are important for channel function.

Introduction

The influenza virus remains an important viral pathogen of significant medical importance. The two predominant types of influenza virus that infect humans are the influenza A and B viruses. At the biochemical level, the influenza A and B viruses both have negative-stranded RNA genomes consisting of eight RNA segments. For the influenza B virus, the genome chain length totals 14,639 nucleotides, whereas, for the influenza A virus (A/PR/8/34), the genome totals 13,588 nucleotides. For both the influenza A and B viruses, RNA segments 1–3 encode the three polymerase proteins, PB1, PB2, and PA, RNA segment 4 encodes hemagglutinin (HA), RNA segment 5 encodes the nucleocapsid protein (NP), RNA segment 6 encodes neuraminidase (NA), RNA segment 7 encodes the matrix protein (M), and RNA segment 8 encodes two proteins, NS1 and NEP, that are translated from unspliced and spliced mRNAs using overlapping reading frames (reviewed in Lamb and Krug, 2001).

Despite these genome RNA segment-coding similarities for proteins with similar functional properties, the genomes of the influenza A and B viruses also differ in coding for additional proteins that distinguish the influenza A and B virus types. For all influenza A viruses, RNA segment 7, in addition to encoding the M protein, also encodes the M2 integral membrane protein (A/M2) (Lamb and Choppin, 1981). The M and A/M2 proteins are translated from unspliced and spliced mRNAs, respectively, using overlapping reading frames (Lamb et al., 1981). In addition, most strains of the influenza A virus (but not all) encode on RNA segment 2, using an overlapping reading frame, an 87-residue protein called PB1-F2, and the available evidence suggests that PB1-F2 is involved in virus-induced apoptosis (Chen et al., 2001). The influenza B virus genome encodes two extra proteins of unknown functions. Influenza B virus RNA segment 6, in addition to coding for the B/NA protein, also encodes via a bicistronic mRNA and using an overlapping reading frame, the 100-residue NB small integral membrane glycoprotein (Shaw et al., 1983; Williams and Lamb, 1989). Influenza B virus RNA segment 7, in addition to encoding the M protein, encodes the BM2 protein. A bicistronic mRNA encodes the M and BM2 proteins using a stop-start of translation and two cistrons in tandem (Horvath et al., 1990).

The influenza A virus A/M2 protein has a proton-selective ion channel activity that is the target of the antiviral drug amantadine (Sugrue et al., 1990; Sugrue and Hay, 1991; reviewed in Hay, 1992; Lamb et al., 1994). The A/M2 protein ion channel activity functions during uncoating of virions in endosomes, permitting protons to enter the virion and causing protein-protein dissociation, principally by weakening interactions between the M protein and NP protein. In addition, the A/M2 protein functions during its own transport through the exocytic pathway, equilibrating the pH of the lumen of the *trans* Golgi network (TGN) with the cytoplasm (reviewed in Hay, 1992; Lamb et al., 1994). Electrophysiological recordings showed directly that the A/M2 protein has a pH-activated, proton-selective ion channel activity that is blocked by amantadine in vivo (Chizhmakov et al., 1996; Mould et al., 2000a, 2000b; Pinto et al., 1992; Wang et al., 1993).

It is commonly anticipated that the influenza B virus requires an ion channel activity to aid virus uncoating in endosomes, particularly as the influenza B virus M protein can be removed from the ribonucleoprotein (RNP) core by low pH (5.5) treatment (Zhirnov, 1992; see below). However, there has been no report of an influenza B virus-specific protein having an ion channel activity in vivo. Recent evidence has shown that the influenza B virus BM2 protein, which is a component of purified virions (Odagiri et al., 1999), is an integral membrane protein that is expressed at the surface of virus-infected cells (Paterson et al., 2003). The available evidence indicates that the vast majority, if not all, of the BM2 protein molecules, like the A/M2 protein (Lamb et al., 1985), adopts an $N_{out}C_{in}$ orientation. Crosslinking studies suggest that the most likely minimal native oligomeric form of BM2 is a tetramer (Paterson et al., 2003). Thus, we investigated whether BM2 has an ion channel

*Correspondence: ralamb@northwestern.edu

[4]These authors contributed equally to this work.

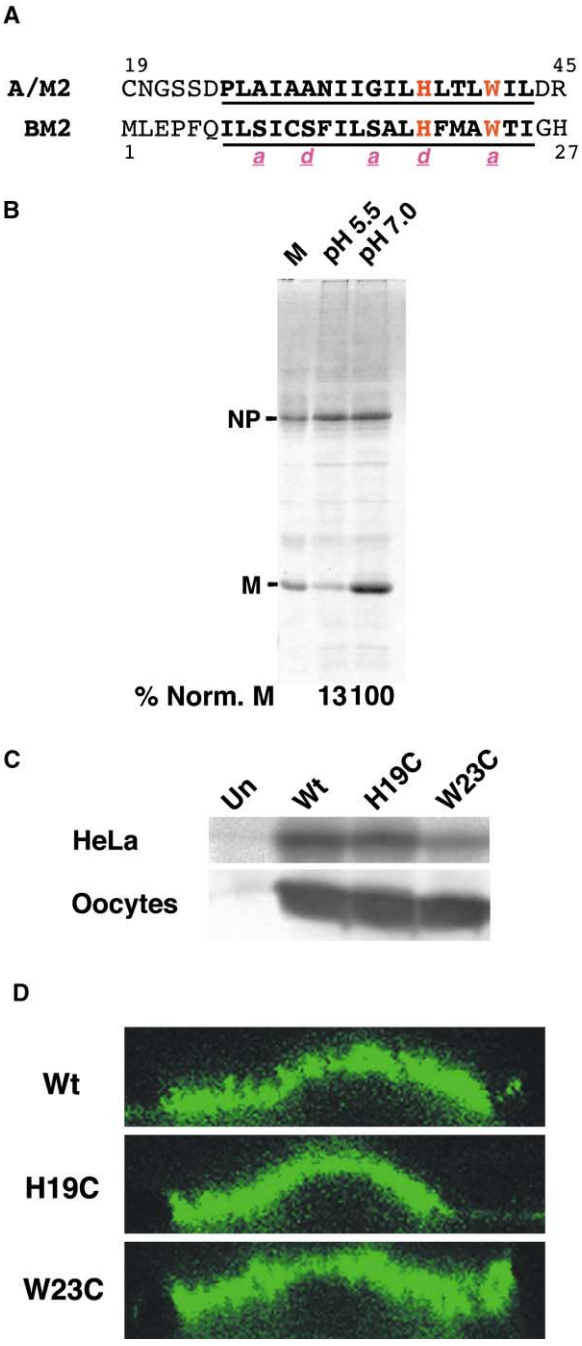


Figure 1. Solubility of the Influenza B Virus M Protein from Purified Virions under Mildly Acidic Conditions and Expression of the Influenza B Virus BM2 Protein and TM Domain Mutants

(A) Comparison of the amino acid sequence of the TM domain and flanking regions of influenza B virus BM2 and influenza A virus A/M2 proteins. The predicted TM residues are shown in bold and underlined, and the conservation of histidine and tryptophan is indicated (red letters). The heptad repeat *a* and *d* residues of a predicted α-helical TM domain are indicated.

(B) Purified egg-grown influenza B virus was treated with 1% Triton X-100 in Mes/Tris buffer at pH 5.5 or pH 7.0 and pelleted through 20% sucrose. The pelleted material was analyzed by SDS-PAGE, and polypeptides were stained with Coomassie brilliant blue. Stained gels were scanned and images were analyzed with an Odyssey infrared imaging system (Li-Cor, Lincoln, NE).

(C) Transient expression of C-terminal epitope-tagged (Flag) wt BM2 and BM2 containing TM domain mutations H19C and W23C in HeLa-CD4-LTR-β-gal cells and *Xenopus* oocytes. Metabolically labeled BM2 was immunoprecipitated from HeLa cell lysates and detected with anti-Flag sera. BM2 in lysates of oocytes was detected by immunoblotting with anti-Flag sera. Un, untransfected cells.

(D) Immunofluorescent staining of epitope-tagged wt BM2, BM2-H19C, and BM2-W23C in HeLa-T4 cells. Z scan reconstructions on serial 7 μm sections in the *x-y* plane were calculated to show surface staining of the proteins.

activity. We were particularly intrigued by the fact that a comparison of the sequence of the TM domain of BM2 and A/M2 indicates that the two key residues for activation and gating, H^{37} and W^{41}, respectively, in the A/M2 ion channel (Tang et al., 2002; Wang et al., 1995) are found at the same spacing, HXXXW (H^{19} and W^{23}), in the BM2 TM domain (Paterson et al., 2003) (see Figure 1A).

Results

Solubility of the Influenza B Virus M Protein from Purified Virions under Mildly Acidic Conditions and Expression of the Influenza B Virus BM2 Protein and TM Domain Mutants

The rationale that the influenza B virus requires an ion channel activity to mediate intravirion acidification during virus uncoating in endosomes was confirmed by showing that low-pH (pH 5.5) treatment of detergent-disrupted purified influenza B virus causes solubilization of the M protein (Figure 1B). In the pellet fraction after pH 5.5 treatment, 13% of the M protein remained as compared to pH 7.0 treatment.

To facilitate testing for BM2 protein ion channel activity, we constructed BM2 mutant proteins (Flag epitope tagged) in which His^{19} and Trp^{23} were replaced by Cys (BM2-H19C and BM2-W23C), as we anticipated that if we found an ion channel activity for wt BM2 then mutation of these residues would lead to altered ion channel activity. The expression of wt BM2 and BM2 mutants in oocytes of *Xenopus laevis* and in mammalian HeLa cells was confirmed (Figure 1C). Surface expression of wt BM2 and BM2 mutants in HeLa cells was demonstrated by immunofluorescent staining and Z scan serial reconstructions of images (Figure 1D).

Expression of BM2 Protein Enables Acidification of Cells

We investigated whether the BM2 protein can cause cytoplasmic acidification. BM2, BM2-H19C, and, as a control, a known proton-selective ion channel, A/M2, were expressed in *Xenopus* oocytes and also in mammalian cells. For oocytes, pH microelectrodes were used to measure the response of intracellular pH to lowering external bathing solution pH (Mould et al., 2000a; Shimbo et al., 1996). Oocytes were bathed in Barth's solution at pH 8.5 and pH 5.8, and the solution was returned to pH 8.5 while the cells were constantly voltage clamped at −20 mV (Figure 2A). Oocytes expressing BM2 acidified when exposed to low extracellular pH with a time course that was faster than that observed for oocytes expressing A/M2. In addition, intracellular

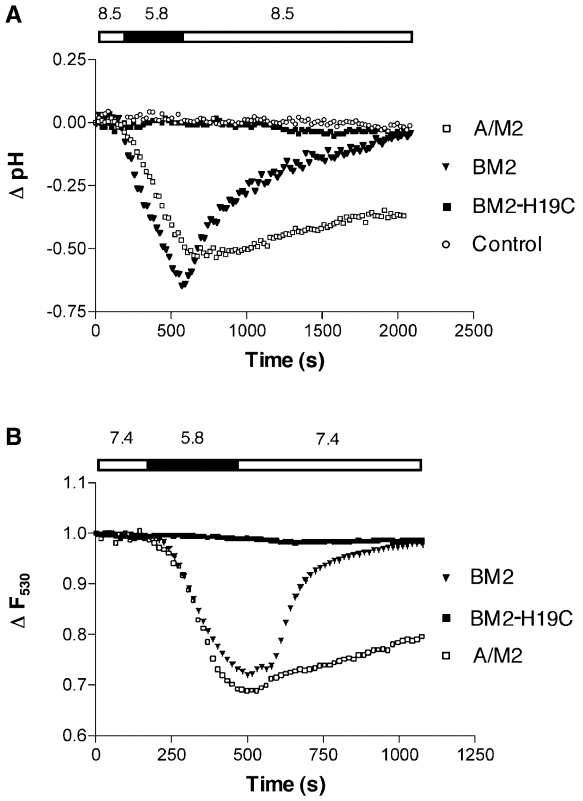


Figure 2. Time Course of Acidification and Recovery from Acidification of BM2 and BM2-H19C in Oocytes and Mammalian Cells

(A) Oocytes expressing wt BM2, BM2-H19C, and A/M2 were bathed in solutions of pH 8.5 or pH 5.8 (at the time shown by the dark bar), and the pH_{in} of these oocytes was measured with a pH microelectrode while the membrane voltage was held at −20 mV with a two-electrode voltage clamp apparatus. Traces are averages from five oocytes.

(B) Time course of acidification of mammalian cells expressing the BM2 protein. HeLa T4 cells were cotransfected to transiently express the BM2, BM2-H19C, or A/M2 proteins together with the EGFP indicator protein. Each of the plots represents the average fluorescence emission at 530 nm of seven cells excited at 485 nm, which has been normalized to the intensity measured initially in pH 7.4 (see Experimental Procedures).

pH of BM2-expressing oocytes fully recovered within 2000 s after return to pH 8.5, unlike the slow and incomplete recovery for the A/M2 proton channel observed during this interval, suggesting a difference in the efflux of protons through the A/M2 and BM2 channels into solutions of alkaline pH. Oocytes expressing BM2-H19C failed to acidify after the external solution pH was lowered, a finding that is consistent with H[19] being important for the function of BM2 ion channel activity and that suggests a possible similarity in ion conduction mechanism between the BM2 and A/M2 proteins.

To show that BM2 exhibited a similar acidification behavior in mammalian cells (and to rule out the unlikely possibility that, in oocytes, BM2 causes upregulation of an endogenous proton channel), we repeated the acidification experiment in HeLa cells (Figure 2B). Here, pH_{in} was monitored by coexpression of EGFP, which exhibits a pH-sensitive fluorescence (Llopis et al., 1998). Consistent with the results obtained in *Xenopus* oocytes, HeLa cells expressing the BM2 protein acidified when the extracellular pH was lowered from pH 7.4 to pH 5.8 and recovered after the bathing solution was returned to pH 7.4. HeLa cells expressing the BM2-H19C mutant protein also failed to acidify under the same conditions. HeLa cells expressing A/M2 acidified after the external pH was lowered but only partially recovered after the bathing solution was returned to pH 7.4.

BM2 Protein Has Ion Channel Activity

Similarities in the acidification profile of cells expressing BM2 and A/M2 in solutions of low pH suggest that BM2 has an ion channel activity. We tested this possibility by using voltage clamp techniques to measure whole-cell membrane currents in *Xenopus* oocytes expressing A/M2, BM2, or BM2 mutant proteins (Flag epitope tagged) in response to lowering bathing solution pH (Figure 3). Oocytes were maintained at pH 8.5 to minimize intracellular acidification, and, at pH 8.5, oocytes expressing the wt BM2 protein had almost no membrane currents at −20 mV. Voltage ramps (−40 mV to + 60 mV) revealed that, at more-positive potentials, BM2-expressing oocytes have a significant outward current, which was larger than that observed for cells expressing A/M2 (Figures 3A, 3C, and 3D; I-V relationship 1). After the external pH was lowered to pH 5.8, BM2-expressing cells developed a large inward current, which closely resembled that observed with cells expressing the A/M2 protein (Figures 3A, 3C, and 3E; I-V relationship 2). The shift in reversal voltage of 83.7 mV ± 9.7 mV (n = 5) is consistent with a shift in the proton equilibrium potential. As only the pH was changed (and the concentrations of other ions were not changed), this suggests that protons are conducted; however, a formal determination of the BM2 ionic selectivity awaits full ion substitution experiments. As we lack an inhibitor of the BM2 ion channel activity, the small endogenous oocyte currents could not be subtracted, and this is one of the reasons why a change in reversal voltage of +58 mV per pH unit for a pure proton conductance was not observed. A second reason is that oocytes expressing proton channels acidify near the plasma membrane while being bathed in a low pH solution (Mould et al., 2000a). Oocytes expressing the BM2-H19C protein did not show pH-activated currents (Figures 3B and 3D–3F), suggesting that this mutation renders the BM2 ion channel inactive. We also tested the ion channel activities of BM2 and BM2-H19C that was not Flag epitope tagged, and the data obtained were indistinguishable from those obtained for the tagged proteins (data not shown).

The current-voltage (I-V) relationships obtained from voltage ramps at pH 5.8 (Figures 3D–3F) indicate that, although the BM2 and A/M2 currents were of similar amplitude at −20 mV, their voltage dependence differs, with BM2-expressing cells showing more current in the outward direction for positive voltages than A/M2-expressing cells. After the pH was returned to 8.5, membrane currents of BM2 protein-expressing cells declined rapidly in amplitude (Figures 3A and 3F; I-V relationship 3). Differences between BM2 and A/M2 channels were observed in the I-V relationship measured after return to pH 8.5 (Figure 3F), which, for oocytes expressing

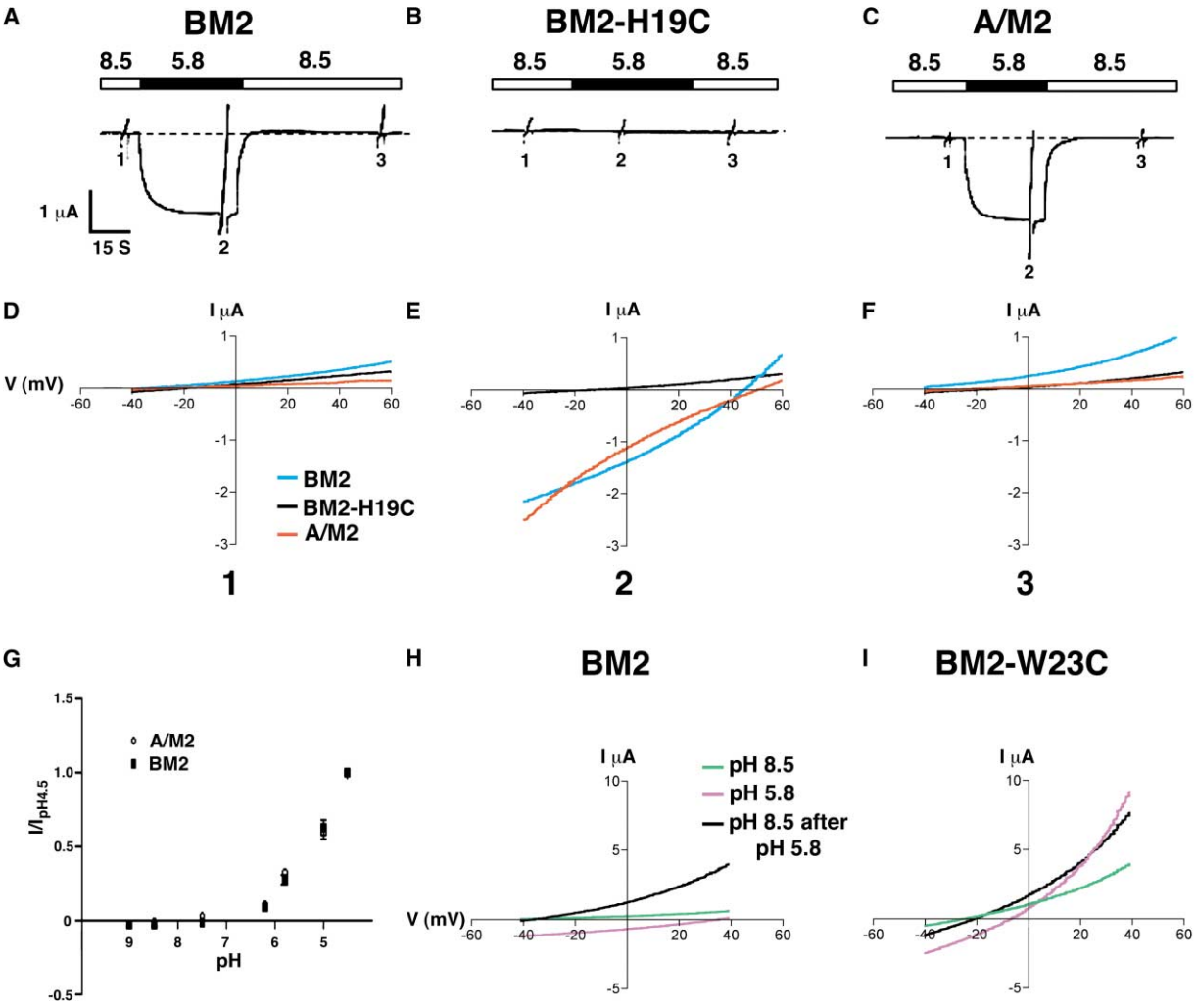


Figure 3. Direct Measurement of BM2 Ion Channel Activity in Oocytes of *Xenopus laevis* by Two-Electrode Voltage Clamp Technique

(A–C) Comparison of whole-cell membrane currents recorded from oocytes of *Xenopus laevis* expressing (A) wt BM2, (B) BM2-H19C, and (C) A/M2. At the time indicated by the dark bar, the pH of the bathing medium was lowered from pH 8.5 to pH 5.8. The membrane voltage was held at −20 mV, except for one occasion while bathed in a solution of pH 5.8 and two occasions while bathed in a solution of pH 8.5; during these times, the membrane voltage was varied continuously from −40 mV to +40 mV.

(D–F) The resulting current-voltage relationship for each ramp designated in (A), (B), and (C) at times indicated by 1, 2, and 3 is plotted on the graphs below the time course records.

(G) Comparison of the current amplitude of A/M2- and BM2-expressing oocytes in solutions of various pH plotted as a fraction of the current amplitude obtained at pH 4.5.

(H and I) Current-voltage relationship of oocytes expressing the wt BM2 (H) and BM2-W23C (I) proteins at pH 8.5, pH 5.8, and pH 8.5 after pH 5.8 treatment. Note the change in scale compared to the curves shown in (D), (E), and (F) and that the voltage ramps extended to only +40 mV to prevent loss of clamp due to the large current amplitudes that were characteristic in oocytes expressing the BM2-W23C mutant.

BM2, showed pronounced outward currents at more positive voltages that were larger than those observed at the same pH before exposure to the pH 5.8 solution. In comparison, A/M2-expressing cells had no outward current at −20 mV and an I-V relationship that was indistinguishable from that of the inactive BM2-H19C mutant (Figure 3F), which is consistent with the A/M2 channel being closed at pH 8.5. Although the BM2 and A/M2 protein-mediated currents showed a slight difference in their voltage dependence, their pH dependence was very similar (Figure 3G), suggesting a common mechanism of pH activation. Treatment of BM2-expressing oocytes with the A/M2 channel activity inhibitor, amantadine (100 μM), had no effect (data not shown), as would be anticipated given the different sequences of the BM2 and A/M2 TM domains and that influenza B virus replication is not inhibited by micromolar concentrations of amantadine (Davies et al., 1964).

Recent data indicate a model of elegant simplicity for A/M2 in which the bulky indole side chain of residue W^{41} regulates the A/M2 channel by functioning as a minimalistic gate that opens and closes the pore (Tang et al., 2002). Thus, given the conservation of spacing of H^{37} and W^{41} in the A/M2 channel and H^{19} and W^{23} in the BM2 channel, we constructed the mutant BM2-W23C. When whole-cell membrane currents were measured for oocytes expressing BM2-W23C, large predominantly outward currents were observed (Figure 3I). The smaller

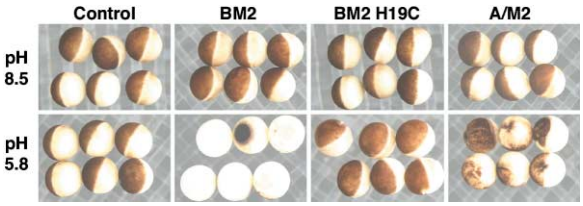


Figure 4. BM2 Ion Channel Activity Correlates with Changes in Oocyte Morphology

Oocytes were microinjected with mRNA for BM2, BM2-H19C, and A/M2 or with water for control oocytes and bathed in pH 8.5 medium for 36 hr. The bathing solution was then replaced with either new solution at pH 8.5 (upper panel) or changed to a bathing solution of pH 5.8 (lower panel), and the oocytes were held for a further 12 hr. Oocytes were then photographed.

change in reversal voltage of oocytes expressing BM2-W23C after lowering the pH of the bathing medium from pH 8.5 to pH 5.8 treatment (10 mV $\pm$ 3.8 mV, n = 5) than for wt BM2 (compare Figure 3H to Figure 3I) suggests that BM2-W23C is less proton selective than wt BM2. The difference between the I-V relationship of oocytes expressing wt BM2 and BM2-W23C measured in pH 8.5 solution after prior bathing in pH 5.8 suggests that BM2 residue W^{23}, like residue W^{41} of A/M2, plays an important, yet subtly different, role in channel gating.

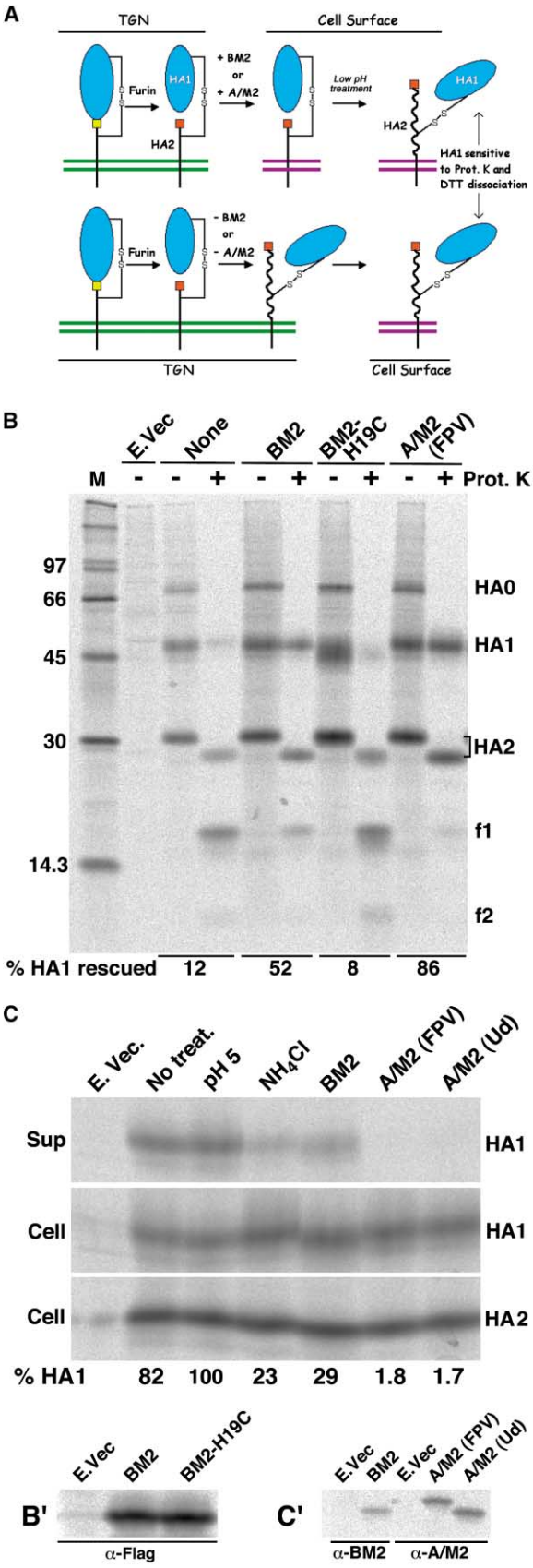
We observed that the morphology of oocytes correlated with their acidification. Although control oocytes can withstand low extracellular pH treatment (pH 5.8) for several days, expression of low-pH-activated wt BM2 (but not BM2-H19C) caused a significant alteration of the morphology of the oocytes at the animal and vegetal pole interface, a sign of impending oocyte death (Figure 4). An assay for discovery of inhibitors of the A/M2 ion channel activity was based on this method (Giffin et al., 1995).

BM2 Protein Expression Stabilizes the Native Form of Hemagglutinin during Intracellular Transport

The influenza HA glycoprotein has two major roles in the influenza virus life cycle. It binds to the virus receptor, sialic acid, and, once the virus is internalized and resident in the lumen of endosomes, HA mediates fusion of the viral envelope with the endosomal membrane. HA is synthesized as an uncleaved precursor (HA0) that is trapped kinetically in a metastable conformation. Cleavage of HA0 to two disulfide-linked chains, HA1 and HA2, occurs either intracellularly or extracellularly, depending on the virus strain. Cleavage causes HA to adopt a new lower-energy metastable conformation (pH 7 HA), which can be triggered to undergo a refolding event by the low pH (pH 5–6) found in endosomes, to form the final most-stable form of HA. This latter refolding event either causes or leads to membrane fusion (reviewed in Skehel and Wiley, 2000). For some subtypes of avian influenza virus, their HA is cleaved intracellularly in the TGN, and a smaller subset of these HAs have a high pH optimum (pH 5.9) of fusion (e.g., fowl plague virus [FPV] Rostock). When FPV HA is expressed from cDNA in the absence of A/M2 expression, it undergoes its conformational change to the low-pH-induced form in the lumen of the TGN (Sugrue et al., 1990; Takeuchi and Lamb, 1994). Coexpression of A/M2 rescues FPV HA in its pH 7 form (Takeuchi and Lamb, 1994) because the A/M2 ion channel activity causes equilibration of the acidic pH of the TGN lumen with the cytoplasm and keeps the pH above the threshold at which the HA conformational change occurs (Figure 5A). The conformation of FPV HA thus provides an assay for BM2 and A/M2 ion channel activity that is independent of other techniques and their possible artifacts. The native form of HA can be distinguished from the low-pH-induced conformation because (1) proteinase K specifically digests the low-pH form, whereas pH 7 HA is relatively protease resistant (Skehel et al., 1982), and (2) reducing agents can reduce the disulfide bond between HA1 and HA2 and cause the release of the HA1 subunit when HA is in the low-pH-induced form (Graves et al., 1983; Takeuchi and Lamb, 1994). The HA of human strains of the influenza A or B viruses cannot be used in this assay, as their pH of transition to the low-pH-induced form ranges from $\sim$pH 5.0–5.3 (Brassard and Lamb, 1997; reviewed in White, 1990).

When FPV HA was expressed alone, labeled metabolically in a pulse and chase protocol (to permit intracellular transport to the Golgi apparatus and cell surface), most of the HA was observed in the cleaved form (HA1 and HA2 subunits) (Figure 5B). Digestion of a cell lysate with proteinase K caused the digestion of much of HA1 to two proteolytic fragments (f1 and f2) and trimming of a terminal segment of HA2, as observed previously (Takeuchi and Lamb, 1994). The small amount of HA0 that was digested by proteinase K treatment most likely represents HA0 that failed to fold and oligomerize correctly (Copeland et al., 1988). Coexpression of A/M2 (Figure 5C) with FPV HA has been shown previously to rescue HA in its native form (Takeuchi and Lamb, 1994), and, as shown in Figure 5B, 86% of HA1 was resistant to proteinase K digestion. Coexpression of BM2 with FPV HA rescued 52% of HA1 in a protease-resistant form, whereas the inactive BM2-H19C protein failed to rescue HA1 from proteinase K digestion. Thus, these data indicate that BM2 can equilibrate the acidic pH of the TGN lumen with the cytoplasm. Note that, in the experiment shown in Figure 5B, BM2 and BM2-H19C were Flag epitope tagged, which provides confirming data that tagging the molecules does not affect their biological activity.

To show further the effect of BM2 expression on stabilizing the pH 7 form of HA during intracellular transport, we used reductive dissociation of HA1 as an assay for the low-pH-induced conformation of HA. Cells transiently expressing HA were metabolically labeled in a pulse label and chase protocol. Cells were then incubated in 20 mM dithiothreitol (DTT), and the supernatants were collected. The cells were lysed, and HA species in the supernatant and cell lysate (as a loading control) were immunoprecipitated and analyzed by SDS-PAGE. As shown in Figure 5D, when HA was expressed alone, 82% of HA1 could be released on DTT treatment (the amount of HA1 released by DTT after treatment of cell surfaces with pH 5 medium to convert HA to the low-pH-induced conformation was taken as 100%). Incubation of cells with 5 mM NH_4Cl, a treatment that increases intracellular pH, reduced the amount of HA1 (23%) that could be released by DTT treatment. Coexpression of BM2 with FPV HA also reduced the amount



of HA1 (29%) that could be released by DTT treatment, and expression of A/M2 (cDNAs from FPV or Udorn subtypes) greatly reduced the amount of HA1 (<2%) released. In neither the proteinase K sensitivity assay nor the reductive dissociation assay can we compare the "specific activity" of BM2 versus A/M2 because the amounts of BM2 and A/M2 proteins expressed would need to be quantified, and different antisera were used to detect each protein.

Overexpression of BM2 Protein Affects Transport through the Golgi Apparatus

We tested whether overexpression of BM2 would perturb Golgi function, as A/M2 leads to a delay in intracellular transport through the Golgi cisternae and dilation of Golgi cisternae, effects similar to that of the Na^+/H^+ ionophore, monensin (Orci et al., 1984; Sakaguchi et al., 1996). As shown in Figure 6A, coexpression of a constant amount of FPV HA and increasing amounts of BM2 resulted in increased accumulation of uncleaved HA0,

Figure 5. The BM2 Ion Channel Activity Rescues Vector-Expressed Fowl Plague Virus (FPV) HA in pH 7 Form

(A) Schematic diagram to illustrate the conformations of HA (FPV) in the Golgi apparatus with (upper panel) or without (lower panel) coexpression of BM2 or A/M2. Uncleaved HA is shown with a yellow fusion peptide. On cleavage activation HA undergoes a conformational change to relocate the fusion peptide (red). The HA1 (blue) and HA2 (black; membrane-attached) subunits are kinetically trapped in a metastable pH 7 form. At low pH (pH 5.9 for FPV HA), the HA2 subunit undergoes a refolding event.

(B) Rescue of FPV HA native form by coexpression of BM2. HeLa-CD4-LTR-β-gal cells were transfected with plasmids to transiently express HA (FPV), BM2 (Flag tagged), BM2-H19C (Flag tagged), or A/M2 (FPV or Udorn) or pairs of the DNAs as indicated. Total DNA was adjusted to 2 μg per dish with pCAGGS empty vector DNA. At 24 hr post transfection, cells were metabolically labeled for 10 min. Cultures were then incubated in pH-chase medium (pH 7.0) for 60 min. Cell lysates were digested with (+) or without (−) proteinase K; HA, BM2, BM2-H19C, and A/M2 were immunoprecipitated with the appropriate antisera; and polypeptides were analyzed by SDS-PAGE. f1 and f2, proteolytic fragments of HA1.

(B′) Immunoprecipitation of BM2 and BM2-H19C from the experiment shown in (B).

(C) Reductive dissociation of HA1 on the surface of cells and stabilizing activity of BM2. Cells were transfected with plasmids to transiently express FPV HA alone or FPV HA with either BM2, A/M2 (FPV), or A/M2 (Udorn). At 24 hr post transfection, where indicated, the medium was adjusted to contain 5 mM NH_4Cl and maintained in further steps. At 25 hr post transfection, all cultures were metabolically labeled with Pro-mix L[^{35}S] in vitro cell labeling mix for 2 hr. Cells were then incubated for 3 min at 37°C with PBS containing 10 mM MES/10 mM HEPES either at pH 5.0 to induce HA to undergo the transition to its low-pH-induced conformation (pH 5 sample) or at pH 7.0 for the remaining samples. Cultures were then incubated for 10 min at room temperature with PBS containing 20 mM dithiothreitol (Graves et al., 1983). Supernatants were collected and clarified by centrifugation (14,000 × g, 10 min); HA, BM2, and A/M2 were immunoprecipitated with the appropriate antisera; and polypeptides were analyzed by SDS-PAGE. E.Vec, empty vector-transfected cells; No treat., cells held at pH 7.0 at all times; pH 5, cells expressing HA treated with pH 5 medium after the labeling period; NH_4Cl, cells expressing HA and incubated with 5 mM NH_4Cl before and during the labeling period; BM2, A/M2 (FPV), and A/M2 (Ud), cells cotransfected to express HA and the indicated BM2 or A/M2 proteins.

(C′) Immunoprecipitation of BM2 and A/M2 (FPV and Udorn) from the experiment shown in (C).

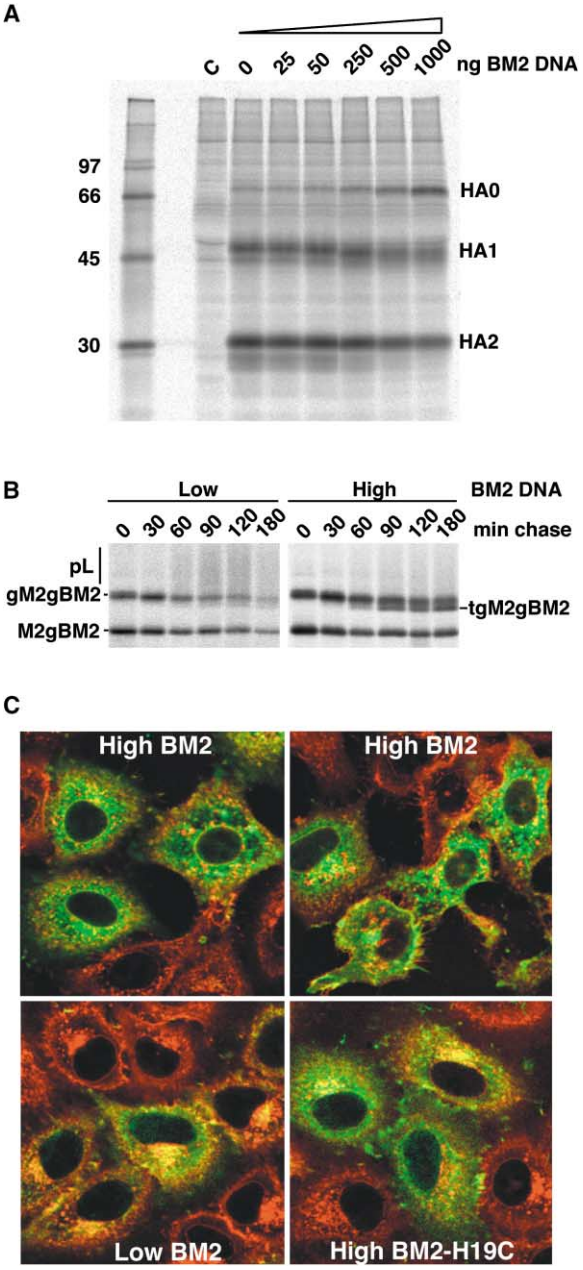


Figure 6. Overexpression of the BM2 Ion Channel Protein Affects Intracellular Transport

(A) Effect of varying the expression level of BM2 on HA cleavage. HeLa-CD4-LTR-β-gal cells were transfected with pCAGGS FPV HA DNA (1 μg) and varying amounts of pCAGGS BM2 (0–1000 ng DNA) and total DNA adjusted to 2 μg with pCAGGS DNA. At 24 hr post transfection, cells were metabolically labeled for 10 min and then incubated in chase medium (pH 7.4) for 60 min. Cells were lysed, HA immunoprecipitated, and then analyzed by SDS-PAGE.

(B) High-level expression of BM2 affects its own transport through the Golgi complex. A modified BM2 protein (M2gBM2) (Paterson et al., 2003) was used, which has the first four residues of BM2 substituted with 25 ectodomain residues (M2g) derived from the ectodomain of a modified A/M2 protein, which contains a site for addition of N-linked carbohydrate. HeLa T4 cells were transfected with 50 ng or 250 ng pCAGGS-M2gBM2, and, at 24 hr post transfection, the cells were metabolically labeled for 10 min and then incubated in chase medium for the times indicated. M2gBM2 was immunoprecipitated with the 14C2 A/M2 ectodomain-specific mAb. M2gBM2 (M_r of ~18 kDa), unglycosylated M2gBM2; gM2gBM2 (M_r of ~22 kDa), M2gBM2 with addition of an N-linked carbohydrate chain to the protein; heterogeneously migrating pL (M_r of ~22–30 kDa), polylactosaminoglycan addition to the high-mannose carbohydrate chain of gM2gBM2; tgM2gBM2, carbohydrate-trimmed form of gM2gBM2.

(C) High-level expression of BM2 ion channel protein disrupts normal Golgi apparatus morphology. HeLa-CD4-LTR-β-gal cells were transfected with 40 ng BM2-Flag DNA (low), 800 ng BM2-Flag DNA (high), or 800 ng BM2-H19C-Flag DNA (high). At 24 hr post transfection, cells were fixed with 2% formaldehyde and permeabilized with 0.5% Triton X-100. Cells were stained with WGA labeled with AlexaFluor 594 to stain for sialic acid and with anti-Flag MAb (M2) and FITC-labeled goat anti-mouse IgG. Red, WGA staining; green, BM2-Flag staining; yellow, merge of red and green coincident staining.

suggesting that either the TGN protease furin is inactivated or that HA0 is slowed in transport to the TGN. HA1 showed a faster gel mobility not normally observed for HA carbohydrate maturation (Sakaguchi et al., 1996) with increasing time of chase, suggesting an alteration in carbohydrate chain modification. Analysis of the kinetics of HA acquiring carbohydrate chains resistant to endoglycosidase H digestion showed a delay on coexpression of BM2 (data not shown). Flow cytometry analysis indicated that, in the DNA concentration range tested, the relative number of expressing cells remained constant as the expression level changed (data not shown). To examine further changes in Golgi function, particularly a delay in intracellular transport, we expressed a modified form of BM2 (M2gBM2) that contains an altered ectodomain (M2g), which contains a glycosylation site in its ectodomain but retains BM2 ion channel activity (data not shown). We have shown previously that the M2g glycosylation site is used and that the attached carbohydrate chain is modified by addition of polylactosaminoglycan (pL) (Paterson et al., 2003; Sakaguchi et al., 1996), a modification that occurs in the Golgi apparatus. As shown in Figure 6B, when low levels (low) of M2gBM2 are expressed (levels similar to those of BM2 found in influenza B virus-infected cells), the protein acquired pL modification with time, whereas, when five times-higher levels of M2gBM2 were expressed (high), pL was not added to M2gBM2 and trimming of carbohydrate chains occurred, as observed when A/M2 protein was overexpressed (Sakaguchi et al., 1996). We examined the morphology of the Golgi apparatus expressing low and high levels of BM2 by immunofluorescent staining. The Golgi was stained with wheat germ agglutinin (WGA) (red) (a lectin that recognizes N-acetyl glucosamine predominantly) and epitope-tagged BM2 with the anti-Flag MAb (green). As shown in Figure 6C, at low levels of BM2 expression or with expression of high levels of the inactive BM2-H19C protein, BM2 staining and WGA staining were largely coincident (yellow) and showed a typical perinuclear Golgi staining pattern. However, high-level BM2 expression resulted in an abnormal pattern with the presence of a large number of vesicles staining green or red and seemingly a smaller number that were coincident. In part this may reflect the concentration of BM2 in the TGN, as found for A/M2 (Sakaguchi et al., 1996). These data are consistent with vesicularization of the Golgi, a process that occurs when the intra-Golgi pH is equilibrated with the cytoplasm because of overexpression

of the A/M2 ion channel protein (Sakaguchi et al., 1996) or monensin treatment (Orci et al., 1984).

Discussion

Recently, it was determined that influenza B virus BM2 is an oligomeric integral membrane protein that is expressed at the cell surface in an $N_{out}C_{in}$ orientation (Paterson et al., 2003). We show here that BM2, when expressed in *Xenopus* oocytes or in mammalian cells, causes acidification of the cells when the external pH is lowered. Electrophysiological recordings demonstrate a pH-activated ion channel that is consistent with the channel being predominantly proton selective, although definitive confirmation of the ionic selectivity remains to be determined. Expression of BM2 in mammalian cells also prevented FPV HA from undergoing its conformational transition to the low-pH-induced form in the Golgi apparatus, data that is consistent with BM2 equilibrating the pH gradient between the Golgi apparatus (lumenal acidic pH) and the cytoplasm (neutral pH). BM2 expression at high levels also causes a delay in intracellular transport in the exocytic pathway and causes morphological changes in the Golgi, consistent with dilation and vesicularization of the cisternae of the Golgi apparatus. Although analysis of the ion channel activity of small integral membrane proteins expressed in oocytes is hampered by the fact that some proteins, including the influenza B virus NB glycoprotein, cause activation of a Cl^- conductance endogenous to oocytes (Shimbo et al., 1995), the different approaches we used to show ion channel activity of BM2 lead us to believe that it is an intrinsic property of the protein. Furthermore, the mutants BM2-H19C and BM2-W23C show activities consistent with these residues lining the aqueous pore of the BM2 channel. In addition, the ion channel properties of BM2 are very similar to, but subtly different from, the well-characterized influenza A virus A/M2 proton-selective ion channel.

For both the influenza A and B viruses, the M protein can be removed from the RNP core by low-pH treatment (Zhirnov, 1992, and Figure 1), and BM2 and A/M2 are minor components of virions (Odagiri et al., 1999; Zebedee and Lamb, 1988). Thus, these ion channel proteins provide the means to acidify the virion interiors when the virions are in the acidic environment of the endosome. Both BM2 and A/M2 are encoded by RNA segment 7 of the influenza B and A viruses, respectively, yet BM2 is encoded by a bicistronic mRNA using a stop-start of translation with dual cistrons in tandem, whereas A/M2 is encoded by a spliced mRNA, with the bulk of the coding region of A/M2 overlapping the M protein reading frame. Despite this dissimilarity in coding mechanism, BM2 contains 109 residues and is oligomeric, most likely a tetramer (Paterson et al., 2003), and the A/M2 ion channel is a homotetrameric integral membrane protein, with each chain of the mature protein containing 96 amino acid residues (Holsinger and Lamb, 1991; Lamb et al., 1985; Sugrue and Hay, 1991; Tobler et al., 1999). Both BM2 and A/M2 have small ectodomains ($\sim$5 residues and 23 residues, respectively) and 19-residue TM domains that contain the conserved HXXXW motif, and these TM domains serve as the proteinaceous core (the channel pore) that allows a flux of protons across the membrane. Thus, this raises the question of whether there was parallel evolution from a common influenza virus progenitor before the separation of the influenza A and B viruses or, alternatively, whether the BM2 and A/M2 proteins represent an example of convergent evolution.

As overexpression of BM2 affects Golgi function, it would be predicted to be deleterious to the normal expression of influenza virus integral membrane proteins at the cell surface. Thus, it would be advantageous to the viruses to control the amount of synthesis of BM2. Synthesis of BM2 in influenza B virus-infected cells is controlled by the extent of translational termination of M protein synthesis and reinitiation of BM2 synthesis (Horvath et al., 1990).

Previously it has been speculated that the influenza B virus NB protein may have ion channel activity. Indeed, when bacterially expressed NB protein was reconstituted into planar membranes, it was reported to have an ion channel activity (Fischer et al., 2001; Sunstrom et al., 1996). However, these planar bilayer experiments are subject to artifacts (reviewed in Lamb and Pinto, 1997). Furthermore, the ion channel activity reported by Fischer et al. (2001) was blocked by amantadine, yet amantadine does not inhibit the replication of the influenza B virus (Davies et al., 1964). Although we observed that NB expression in oocytes upregulates an endogenous Cl^- conductance (Shimbo et al., 1995), we could not demonstrate acidification of oocytes on NB expression and lowering external pH (our unpublished data).

The most striking functional difference between the A/M2 and BM2 ion channel proteins lies in their sensitivity to amantadine. One model for the inhibition of the A/M2 channel by amantadine shows an amine hydrogen serving as a surrogate proton coordinating with an imidazole nitrogen of histidine (Pinto et al., 1997). This raises the question of why the BM2 channel is not inhibited by amantadine by similar coordination with TM domain His^{19}. One likely reason is that the pore-lining residues facing the ectodomain differ between the two proteins (see Figure 1A). This explanation is supported by the finding that amantadine-resistant mutations are found in these pore-lining residues of A/M2 (Hay et al., 1985). Interestingly, for BM2, three serine residues are found on the same face of the TM domain putative α helix as H^{19} and W^{23}, thus providing an aqueous pore lining. A second functional difference between BM2 and A/M2 lies in the ability of BM2 to conduct ions while its ectodomain is bathed in solutions of high pH (Figures 3H and 3I). This may reflect a difference in the nature of the interactions of the His and Trp residues in the BM2 and A/M2 TM domains in how they close the pore at high pH (Tang et al., 2002). The findings that BM2 and A/M2 have distinct TM domains that leads to largely proton-selective ion channels with similar functions for two related, but separate, viruses raise interesting questions in understanding protein structure and design.

Experimental Procedures

Cells and Influenza B Virus Growth and Purification

HeLa T4 cells and HeLa CD4-LTR-β-gal cells were grown and maintained as described previously (Paterson et al., 2003). The influenza B virus (B/MD/59) was grown in 10-day-old embryonated chicken eggs and purified on sucrose gradients (Paterson and Lamb, 1993).

Plasmids, Mutagenesis, and Transfections

For synthesis of synthetic mRNAs, the BM2 coding region (Horvath et al., 1990; Paterson et al., 2003) was cloned into pGEM3 (Promega, Madison, WI.). The expression vector pCAGGS (Niwa et al., 1991) was used for transient expression of BM2, BM2 mutants, A/M2 (derived from the influenza virus fowl plague virus [FPV] A/chicken/Germany/34 or A/Udorn/72), or FPV HA cDNAs in tissue culture cells. Details of all cloning steps are available upon request. Site-specific mutagenesis was performed by the four-primer PCR method. pEGFP was obtained from Clontech (Palo Alto, CA). The nucleotide sequence of the entirety of the BM2 coding region was determined. Transfections of HeLa T4 cells or the highly transfectable HeLa CD4-LTR-β-gal cells was done with Lipofectamine-PLUS (Invitrogen) or Trans IT LT-1 (Panvera, Madison, WI) following the manufacturer's recommendations.

Antibodies

Anti-Flag MAb (M2) was obtained from Sigma-Aldrich (St. Louis, MO). The MAb14C2 (M2tag) has been described previously (Zebedee and Lamb, 1988). HA protein was immunoprecipitated with a rabbit antibody prepared against an inactivated H7N1 avian influenza virus. WGA was labeled with Alexafluor 594 (Molecular Probes, Eugene, OR).

Metabolic Radiolabeling, Immunoprecipitation, Immunoblotting, and SDS-PAGE

Cells were metabolically labeled with Pro-mix L[^{35}S] in vitro cell labeling mix (Amersham Biosciences, Piscataway, NJ). For immunoprecipitation from transfected cells, lysates were prepared in RIPA buffer (Paterson and Lamb, 1993), and proteins were immunoprecipitated with appropriate antibodies. For the detection of BM2 expression in oocytes, immunoblotting was performed as described previously (Holsinger et al., 1994). BM2 was detected with anti-Flag sera and the ECL+ kit (Amersham Pharmacia, Piscataway, NJ), and chemiluminescence was detected with a Storm System Phospholmager (Molecular Dynamics, Sunnyvale, CA). Radioactivity was quantified with a Fuji BioImager 1000 and Mac Bas software (Fuji Medical Systems, Stamford, CT).

Immunofluorescent Staining of BM2 Protein

HeLa T4 cells were transfected with the appropriate plasmids, fixed, permeabilized, and incubated with the appropriate primary antibodies as described previously (Paterson et al., 2003). Fluorescence was observed with a Zeiss LSM 410 confocal microscope (Zeiss, Thornwood, NY) and Zeiss software.

In Vitro Synthesis of mRNA Transcripts

For in vitro transcription, pGEM3BM2 was linearized downstream of the T7 DNA-dependent RNA polymerase promoter and the BM2 cDNA with HindIII. In vitro synthesis of mRNA was carried out as described previously (Pinto et al., 1992).

Culture and Microinjection of Oocytes

Oocytes were removed from female *Xenopus laevis* (Nasco, Fort Atkinson, WI), defolliculated, and maintained at 19°C as described previously (Shimbo et al., 1996). Oocytes at stage V were microinjected with 50 nl of mRNA (1 ng/nl) on the day after defolliculation, incubated for 24 hr in ND96 (pH 7.5) (Pinto et al., 1992), and, finally, incubated for 24 hr in ND96 (pH 8.5) at 19°C before use.

Measurement of Membrane Current of Oocytes

Whole-cell currents were measured with a two-electrode voltage clamp as described previously (Pinto et al., 1992). Continuous current-voltage (I-V) relationships were measured with ramps of membrane voltage, as the BM2 channel shows no rapid voltage or time-dependent gating. These ramps typically spanned a range of 120 mV in 2 s. Oocyte-holding potential was −20 mV, unless stated otherwise.

Measurement of pH_{in} of Oocytes and HeLa Cells

pH microelectrodes were used to measure the internal pH of oocytes voltage clamped at −20 mV as described previously (Mould et al., 2000a). pH-dependent changes in the fluorescence intensity of EGFP were used to monitor changes of pH_{in} of HeLa cells expressing the wt BM2 and BM2 mutant proteins. Briefly, HeLa cells were cotransfected with plasmids encoding EGFP and BM2 two days prior to experimentation. Transfected cells on glass coverslips were transferred to a 2 ml recording chamber (Axon Instruments, Foster City, CA) mounted onto the stage of an epifluorescence microscope equipped with a ×40, 1.0 NA (Zeiss) oil immersion objective that allowed several cells to be visualized in its field at one time. Cells were bathed constantly at a flow rate of 1 ml/min in a solution containing 140 mM NaCl, 5.3 mM KCl, 0.55 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 15 mM HEPES (pH 7.4) or 15 mM MES (pH 5.8) at an osmolality of 205–215 osmol/kg. EGFP-expressing cells (n = 7) were illuminated at 485 nm, and emission intensity was detected at 530 nm with an intensified CCD camera (Photon Technology International, London, Ontario, Canada). The pH dependence of EGFP fluorescence in HeLa cells (n = 14) was qualitatively determined initially in non-BM2-expressing cells by exposure to solutions of five different pH values, from 9.0 to 4.0, in the presence of the protonophore FCCP (20 μM). Fluorescence intensity over the time course of the experiment was normalized for each EGFP-expressing cell to the initial value obtained at pH 7.4.

Acknowledgments

This research was supported in part by research grants R37 AI-20201, RO1 AI-23173 (RAL), and AI-31882 (LHP) from the National Institute of Allergy and Infections Diseases. M.T. is an associate and R.A.L. is an Investigator of the Howard Hughes Medical Institute.

Received: March 6, 2003
Revised: April 8, 2003
Accepted: April 17, 2003
Published: July 7, 2003

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