Reduced incorporation of the influenza B virus BM2 protein in virus particles decreases infectivity

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Abstract

BM2 is the fourth integral membrane protein encoded by the influenza B virus genome. It is synthesized late in infection and transported to the plasma membrane from where it is subsequently incorporated into progeny virus particles. It has recently been reported that BM2 has ion channel activity and may be the functional homologue of the influenza A virus M2 protein acting as an ion channel involved in viral entry. Using a reverse genetic approach it was not possible to recover virus which lacked BM2. A recombinant influenza B virus was generated in which the BM2 AUG initiation codon was mutated to GUG. This decreased the efficiency of translation of BM2 protein such that progeny virions contained only 1/8 the amount of BM2 seen in wild-type virus. The reduction in BM2 incorporation resulted in a reduction in infectivity although there was no concomitant decrease in the numbers of virions released from the infected cells. These data imply that the incorporation of sufficient BM2 protein into influenza B virions is required for infectivity of the virus particles.

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Introduction

Influenza viruses are important human pathogens that have been prevalent throughout the entire world for centuries. Influenza viruses are within the family Orthomyxoviridae and are classified as either influenza virus type A, B, or C. Influenza A virus was first isolated in 1933 (Smith et al., 1933). In 1940, an influenza virus that had no antigenic relationship to influenza A virus was isolated and termed influenza B virus (Horsfall et al., 1940). Both influenza A and B viruses are negative-stranded RNA viruses containing eight gene segments that encode proteins of similar structure and function. Segments 1–3 encode the polymerase proteins, PB1, PB2, and PA. Segment 4 encodes the hemagglutinin protein HA; segment 5 encodes the nucleoprotein NP; segment 6 encodes the neuraminidase enzyme NA; segment 7 encodes the matrix protein M1; and segment 8 encodes two proteins, the nonstructural protein NS1 and nuclear export protein NEP.

Despite these similarities, influenza viruses contain proteins unique to either type A or type B viruses. For example, segment 2 of some influenza A viruses encodes the PB1-F2 protein from a reading frame overlapping that of the PB1 gene. PB1-F2 is thought to be involved in virally induced apoptosis (Chen et al., 2001), but the ORF is not seen in any influenza B viruses. Secondly, whereas segment 6 of influenza A viruses encodes only one protein, NA, segment 6 of influenza B viruses also encodes the NB protein (Shaw et al., 1983) from an open reading frame initiated four nucleotides before the AUG for NA translation (Williams and Lamb, 1989). NB is a 100 amino acid protein that is incorporated into virions (Betakova et al., 1996; Brassard et al., 1996). It has a single hydrophobic region that anchors the protein in the membrane in a NoutCin orientation and lacks a cleavable signal sequence, classifying it as a type III integral membrane protein. The function of NB remains unknown; however, it has recently been found that NB is not essential for the production of viable virus in tissue culture (Hatta and Kawaoka, 2003; DJ and WB unpub-
lished data). It has been proposed that NB has ion channel activity (Chizhmakov et al., 1998; Fischer et al., 2001; Sunström et al., 1996).

Further genetic differences are found between the influenza type A and type B segment 7 RNAs. Thus, although both virus types encode a second protein from this RNA in addition to the M1 major structural gene, the M2 and BM2 proteins are produced using quite different coding strategies. The influenza A virus M2 protein is translated from a spliced mRNA (Inglis and Brown, 1981; Lamb and Choppin, 1981; Lamb et al., 1981), a process controlled by the cellular SR splicing factor SF2/ASF (Shih and Krug, 1996). M2 is a 96 amino acid integral membrane protein expressed abundantly at the infected cell surface and incorporated at lower levels into virions (Jackson et al., 1991; Zebedee and Lamb, 1988). M2 forms a tetrameric channel (Holsinger and Lamb, 1991; Sugrue and Hay, 1991) with ion channel activity (Pinto et al., 1992; Wang et al., 1994) that can be blocked by the anti-influenza drug amantadine (Hay, 1992; Pinto et al., 1992; Wang et al., 1993). The M2 ion channel has two functions (reviewed by Hay, 1992): firstly, it allows the passage of protons from the endosome into the virion core upon viral entry. This acidification leads to the dissociation of vRNA complexes from the M1 protein allowing their transport into the cytoplasm and ultimately to the nucleus for replication. Secondly, M2 equilibrates the pH within the trans-Golgi network (TGN) (Grambas and Hay, 1992; Grambas et al., 1992). This facilitates the transport of HA proteins containing multi-basic cleavage sites to the cell surface in their native form as it prevents their low-pH conformational change (Ohuchi et al., 1994; Takeuchi and Lamb, 1994). The 19 amino acid transmembrane (TM) domain forms the ion channel (Holsinger et al., 1994; Pinto et al., 1992; Wang et al., 1993). Two key residues in the TM domain are H37, which is thought to become protonated under low pH out conditions leading to the activation of the channel (Mould et al., 2000; Wang et al., 1995), and W41, which acts as the channel gate (Tang et al., 2002).

The second protein encoded on influenza B virus RNA segment 7 is BM2, a 109 amino acid type III integral membrane protein that, like influenza A virus M2 protein, is expressed at the cell surface and incorporated into virions (Odagiri et al., 1999; Paterson et al., 2003; Watanabe et al., 2003). BM2 is encoded from the second open reading frame of a bicistronic mRNA with the capacity to encode a 195 amino acid protein (Briedis et al., 1982; DeBorde et al., 1988; Hiebert et al., 1986). Translation is thought to occur via a coupled termination and reinitiation process around the BM1 stop codon via a UAAUG pentanucleotide motif (Horvath et al., 1990). Interestingly, the BM2 TM domain also contains a HXXXW motif reminiscent of that seen for influenza A virus M2. It has recently been shown that BM2 expressed in Xenopus oocytes possesses ion channel activity and that H19 and W23 are required. Furthermore, in mammalian cells, BM2 expression mediated a change in cytoplasmic pH when external pH was lowered and also affected the pH of the TGN (Mould et al., 2003). That influenza B virus requires an ion channel activity like that of influenza A virus was implied by the observations that low pH can dissociate M1 from RNP (Mould et al., 2003; Zhirnov, 1992). Thus, BM2 is likely to be the influenza B virus functional homologue of the influenza A virus M2 protein (Mould et al., 2003).

We have used a reverse genetics system recently established in our laboratory for the rescue of recombinant influenza B viruses (Jackson et al., 2002) to study the role of the BM2 protein in the context of a viral infection. We have attempted to generate recombinant viruses which either lack the BM2 protein altogether to assess whether it is essential for the production of viable virus, or that have reduced levels of translated BM2 to assess at which stage of viral infection BM2 is required. If BM2 is indeed an ion channel, then we hypothesized that a reduction in BM2 translation may reduce the efficiency of viral entry.

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Fig. 1. Sequence changes introduced into the pPRM plasmid via site-directed mutagenesis. pPRBM2ss1 mutations were designed to reduce BM2 translation, pPRBM2sst mutations were designed to knockout BM2 altogether. Nucleotides 769–791 of the influenza B virus segment 7 are shown, as are the amino acids forming the N-terminus of BM2 translated from the +2 reading frame. Stop codons are underlined, nucleotide mutations are in bold, BM2 initiation codon in larger type.
Thus viral particles deficient in BM2 should show a decreased infectivity compared to the isogenic wild-type virions.

Results

Generation of a recombinant virus containing mutations within the BM1–BM2 translation termination initiation motif

Initiation of BM2 translation occurs via a termination reinitiation motif (UUAUG) spanning nucleotides 769–773 around the BM1 termination codon. We attempted to knock out the BM2 protein using an established influenza B virus reverse genetics system (Jackson et al., 2002). We mutated this region of the B/Beijing/1/87 virus vRNA segment 7 sequence within the pPRM rescue plasmid to remove the BM2 initiation codon and to introduce two tandem stop codons downstream in frame with the BM2 ORF (Fig. 1). The resultant plasmid, pPRBM2stop, was co-transfected into 293T cells with four ‘helper’ plasmids which direct expression of the influenza B viral polymerases and nucleoprotein. As a control, the wild-type pPRM plasmid was also transfected with the helper plasmids. Western blot analysis of the resulting BM2-expressing cells revealed three bands specific to BM2 migrating with molecular weights of 12, 17, and 24 kDa. The predicted molecular weight for BM2 is 12 kDa. The
identity of the 17-kDa species has been suggested by Odagiri et al. (1999) to be a phosphorylated form of BM2. We suggest that the 24-kDa species is a BM2 dimer. In contrast, no BM2 protein production was detected as a result of the BM2 stop mutations (Fig. 2). Plasmid pPRBM2stop was then used in place of wild-type pPRM plasmid in the influenza B virus rescue system. However, on repeated occasions when wild type virus Brec was recovered with high efficiency, virus rescue was unsuccessful. Therefore, it is likely that the BM2 protein is essential for the production of viable virus.

We then replaced the BM2 initiation codon AUG with GUG (Fig. 1). The aim was to reduce the amount of BM2 protein translated. To ensure translation of BM1 did not run through the UAG stop codon, a second termination signal was introduced directly after the first by replacement of a C with an A at the +4 position of the BM2 gene. This also resulted in a coding change to the second codon of BM2 from Leu to Ile. The resultant plasmid pPRBM2ss1 was co-transfected into 293-T cells with the four plasmids expressing the viral polymerase and nucleoprotein. Western blot analysis demonstrated the production of BM2 within transfected cells although at a lower concentration than BM2 protein expressed from the pPRM wild-type plasmid (Fig. 2). The pPRBM2ss1 plasmid was then used in the rescue system in place of wild-type pPRM plasmid. A recombinant virus designated BM2ss1 was rescued. RNA segment 7 of the recombinant virus BM2ss1 was fully sequenced to confirm the presence of the desired mutations and the absence of nonspecific changes in the RNA. Western blot analysis was performed on infected Madin–Darby canine kidney (MDCK) cell lysates to determine the efficiency of BM2ss1 BM2 protein translation compared to that of wild-type virus. Fig. 3C shows that BM2 protein concentration in cells at 10 h post-infection is significantly reduced whereas similar concentrations of NP and M1 were present in each infected cell lysate (Figs. 3A and B). Following lysate titration, we estimate the reduction in BM2 to be approximately 8-fold in BM2ss1-infected cells when compared to Brec-infected cells (data not shown). By 24 h postinfection, the levels of BM2 protein accumulated within BM2ss1-infected cells had attained the same levels as those seen in Brec-infected cells (Fig. 3C).

BM2 protein concentration was also determined in concentrated samples of wild-type and BM2ss1 virions harvested 48 h postinfection. There was an approximate 8-fold decrease in BM2 protein concentration in BM2ss1 virions when compared with wild-type virus particles (Fig. 3D) whereas similar NP concentrations were observed (Fig. 3E). Therefore, not only is BM2 present at a reduced level in

![Fig. 4. (A and B) Immunofluorescence analysis of Brec and BM2ss1-infected MDCK cells. Cells were infected with either virus at an MOI of 0.5 and fixed or permeabilized in methanol/acetone 10 h postinfection. (A) Cells were stained with a rabbit anti-BM2 antibody and a goat anti-rabbit FITC-conjugated secondary antibody. (B) Cells were stained with a mouse anti-HA monoclonal antibody and a goat anti-mouse FITC-conjugated secondary antibody. (C and D) Flow cytometry analysis of Brec- and BM2ss1-infected Vero cells. Cells were infected with either virus at an MOI of 1 and harvested in PBS/EDTA 10 h postinfection. Cells were stained with (C) a mouse anti-HA monoclonal antibody followed by a goat anti-mouse FITC-conjugated secondary antibody or (D) a rabbit anti-NB antibody followed by a goat anti-rabbit FITC-conjugated secondary antibody. Cells were fixed in 4% paraformaldehyde and subjected to flow cytometry. Mean fluorescence intensity (MFI) was determined using the WinMDI 2.8 software.](image-url)
BM2ss1-infected cells, it is also incorporated at lower concentrations within virions when compared to wild-type virus.

The effect of decreased BM2 translation on the transport and distribution of HA within infected cells

MDCK cells were infected with \(5 \times 10^5\) pfu/ml (MOI of 0.5) Brec or BM2ss1 virus. Ten hours post-infection, cells were fixed in methanol/acetone (1:1) and used in immunofluorescence assays to study the distribution of BM2 within infected cells. Brec-infected cells showed the presence of BM2 within the cytoplasm and a high level of staining within the Golgi apparatus (Fig. 4A). BM2ss1-infected cells also showed accumulation of BM2 within the Golgi but cytoplasmic staining was reduced in comparison to Brec-infected cells. Thus, this assay also demonstrates the reduction in BM2 translation within BM2ss1-infected cells. Staining with an anti-HA monoclonal antibody showed that both Brec- and BM2ss1-infected cells demonstrated a similar distribution of HA protein within the cytoplasm and at the cell surface (Fig. 4B). In a more quantitative approach, the cell surface expression of HA and also of NB was measured by FACS analysis of Vero cells infected at an MOI of 1 with either wild-type or mutant virus. Interestingly, both proteins showed enhanced cell surface expression in cells infected with the mutant virus (Figs. 4C and D). These results suggest that the transport of the influenza B virus HA protein to the cell surface does not rely on BM2 protein concentration and may even be modified negatively by BM2. They also suggest that the reduced incorporation of BM2 into BM2ss1 virions is not due to a decrease in HA protein at the cell surface.

BM2ss1 virus is attenuated in single step growth kinetics

To further characterize the BM2ss1 virus, its multistep growth kinetics were analyzed. MDCK cells were infected with BM2ss1 or Brec viruses in triplicate at an MOI of 0.001 and released viruses in supernatants harvested every 12 h for 3 days. The samples were titrated via plaque assay to determine the infectious titer of each virus (pfu/ml) and by hemagglutination assay to determine the relative numbers of particles released (HAU/50 μl). Both viruses obtained a peak viral titer at 48 h post-infection, but the titer of BM2ss1 virus was slightly lower (Fig. 5A). However, there were similar numbers of viral particles present in the samples detected by hemagglutination assay with a larger difference in particle/infecitivity ratio noted at the earlier time points. Next, a single step growth curve assay was performed (Fig. 5B). MDCK cells were infected with Brec or BM2ss1 in triplicate at an MOI of 5 and samples harvested every 3 h post-infection. The BM2ss1 infectious titers were reduced 50-fold when compared with wild-type virus, whereas hemagglutination assays showed that both viruses contained the same number of viral particles at each time point. These results suggest that a decrease in BM2 concentration within BM2ss1 viral particles leads to a decrease in viral infectivity.

A decreased concentration of BM2 protein within the virion reduces the efficiency of infection

Influenza B virus nucleoprotein begins to accumulate to high levels within the nucleus of infected cells 4–5 h post-infection and can be detected using an anti-NP primary antibody. By measuring the number of NP positive cells at a specific early time point (e.g., 5 h post-infection), one can determine how efficiently a virus establishes infection in the monolayer. This method was used to compare the efficiency of infection by BM2ss1
and Brec virions. It was important for this experiment to standardize virus input by HA titer rather than by infectious titer to ensure that the relative infectivity of wild-type and mutant virus particles would be measured. Thus, BM2ss1 used in these experiments had an equal HA titer to Brec, but its titer as determined by plaque assay was 10-fold lower. MDCK cells were infected with a range of dilutions of each virus. At 5 h postinfection, the cells were fixed in methanol/acetone (1:1) and stained for NP. At each MOI, there is approximately a 4-fold reduction in the number of cells staining positive following BM2ss1 exposure relative to the wild-type virus (Fig. 6). For example, at an MOI of 0.01 (standardized relative to wild-type virus), 56 Brec-infected cells were visualized compared to 13 BM2ss1-infected cells. The same result was achieved in immunofluorescence assays. The number of infected cells for each virus dilution in both assays is presented in Table 1. The data demonstrate that BM2ss1 virions initiated infection less efficiently than wild-type virus.

### Discussion

The influenza B virus BM2 protein is a type III integral membrane protein that is expressed at the cell surface (Paterson et al., 2003; Watanabe et al., 2003) and is incorporated into virions (Odagiri et al., 1999). There are many similarities between BM2 and the influenza A virus M2 protein such as protein size, membrane topology, and a HXXXW motif in the TM domain (Paterson et al., 2003). The ion channel activity of the influenza A virus M2 protein is involved in viral entry and equilibrating the pH of the trans-Golgi network (TGN). Recent work has shown that BM2 expressed in *Xenopus* oocytes possesses ion channel activity (Mould et al., 2003). Our results are consistent with a role for the BM2 protein as an ion channel required during viral entry. However, we have not excluded the possibility that the deficiency in BM2 results in a defect in the packaging of viral RNA segments leading to a high proportion of particles that lack the full genome complement. Further experimentation to clarify this issue is ongoing.

We were unable to isolate a virus which lacked BM2 protein altogether. Because the reverse genetics system we have developed routinely allows the recovery of $10^8$ pfu/ml recombinant virus at 7 days post-transfection (Jackson et al., 2002), the inability to recover a BM2 knockout virus implies that the production of BM2 protein is essential for virus replication. It is possible that BM2 protein itself is not required for infectivity, but that the 'stop' mutation has affected a cis-acting RNA signal. In the present study, we did not test whether the knockout mutation could be complemented by an exogenous source of BM2 protein.

<table>
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<tr>
<th>Virus</th>
<th>MOI 0.01</th>
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<tr>
<td>Brec</td>
<td>56, 55</td>
<td>10, 9</td>
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<td>BM2ss1</td>
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because we were unable to isolate a cell line expressing BM2. Several groups have addressed the requirement for influenza A virus M2 protein using a reverse genetic approach. However, it should be noted that due to the coding strategy for M2, in none of these approaches has production of an M2-related protein been absolutely prevented. Complete deletion of M2 would demand changes to the M1 open reading frame as the first nine codons of M1 and M2 overlap. However, recombinant viruses in which the M2 transmembrane domain is either completely lacking, replaced by heterologous sequence, or harbors deletions known to abrogate ion channel activity have been described (Takeda et al., 2002; Watanabe et al., 2001). Such viruses were debilitated in growth at early time points and in vivo in mice. The results suggest that M2 is important for efficient viral replication in a single cycle but that an ion channel may not be absolutely essential for virus viability in tissue culture. It was suggested that in the absence of M2, there may be another way for protons to enter the virion, perhaps through pores generated by fusion of HA with the virus membrane. Why this mechanism would not also compensate for influenza B viruses lacking a BM2 ion channel is not clear.

Although complete removal of BM2 did not cause viable virus, a recombinant virus in which BM2 protein was substantially reduced was rescued. This was achieved by mutation of the BM2 AUG initiation codon to GUG, which is used for translation initiation infrequently in Escherichia coli (Kozak, 1983) and occasionally in eukaryotes (Abramczyk et al., 2003; Boeck et al., 1992; Fuxe et al., 2000). Efficiency of initiation from GUG is usually lower than from an AUG in the same context (Kozak, 1989). We estimate that in the context of the virus infection 4- to 8-fold less BM2 was translated from the BM2ss1 segment 7 mRNA. Although this suggests a rather high frequency for translation initiation from the non-canonical codon, it should be borne in mind that the GUG codon is being used in the unusual context of coupled termination–reinitiation and as such may not be subject to the regular rules for the initiation process. The accumulation of M1 protein in infected cells was not affected by the engineered mutation so any phenotype of the mutant virus could be directly attributed to a deficit in BM2 expression.

Purified virions of the mutant virus also incorporated approximately 8-fold less BM2 than wild-type virus. These data might be helpful in understanding how BM2 is recruited into the virion during assembly. For influenza A virus M2, Park et al. (1998) have suggested that incorporation is specific and occurs via an interaction of the M2 ectodomain with another viral component, possibly HA. If this were also the case for influenza B viruses, then the low level of BM2 protein in BM2ss1 mutant virions might be accounted for by a decrease in the level of HA available for assembly at the cell surface. However, analysis of protein expression in cells infected with BM2ss1 virus showed that although intracellular accumulation of BM2 was reduced at 10 h postinfection when compared to wild-type virus, levels of cell surface HA were actually increased, as were levels of NB. One possible explanation for this observation would be that wild-type levels of BM2, like influenza A virus M2, interfere with trafficking of membrane proteins due to the distension of the Golgi apparatus which results from deregulation of the internal organelle pH (Sakaguchi et al., 1996).

In cells infected with mutant virus, the decrease in BM2 expression may be sufficient to significantly reduce or even abrogate this effect. The decrease in BM2 levels in virions could imply that BM2 is passively included in virions rather than actively recruited by another viral protein. Thus, lowered levels in the cell are directly reflected by lower incorporation into virions. Clarification of the mechanism for BM2 incorporation awaits further experimentation.

The ss1 mutation had a significant effect on viral replication which was particularly apparent during the early release phase of progeny virions. Although it is possible that the Leu to Ile change at residue 2 of the BM2 protein affected BM2 activity, it is more likely that the mutant phenotype was due to the reduction in BM2 incorporated into virions. At 10 h postinfection when there was an 8-fold reduction in BM2 expression in infected cells, there was up to a 50-fold reduction in the infectivity of released particles. Later, between 24 and 36 h postinfection, when levels of BM2 were comparable in mutant and wild-type virus-infected cells, BM2ss1 infectivity increased to reach levels only half a log less than for wild-type virus. Thus most of the virus particles produced in the first few hours of budding where BM2 is limited may not incorporate enough BM2 for progeny virus particles to be infectious. Influenza A virus particles contain between 14 and 68 incorporated M2 molecules (Zebedee and Lamb, 1988). We estimate there to be between 20 and 50 molecules of BM2 in each influenza B virus particle (WB, unpublished data). Thus the BM2 incorporation level in wild-type virus is probably already close to minimal. Interestingly, the number of virus particles released was not affected by the decrease in BM2 concentration at the infected cell surface. Hemagglutination assay (HA) results demonstrated a similar number of viral particles were released from cells infected with either virus at all time points. This suggests that a decrease in BM2 levels has little effect on viral assembly and release. In contrast, a reduced cell surface expression of M2 resulting from treatment with a monoclonal antibody caused a reduction in the number of progeny virions released (Hughey et al., 1995).

If BM2 is an ion channel involved in viral entry, a decrease in BM2 incorporation within the virion should lead to a decrease in the efficiency of viral entry. Indeed, in the present study, an immunohistochemical assay which measured early events in infection detected a 4-fold reduction in the number of cells infected with BM2ss1 compared to wild-type virus. A similar result was achieved by measuring the incorporation of radiolabelled amino acids into viral proteins from 2 to 10 h postinfection (data not shown).
Wild-type virus-infected cells contained larger quantities of viral proteins such as NP, HA, and NS1 than BM2ss1-infected cells at each time point, indicating that wild-type viral entry is more efficient.

If BM2 is an ion channel, then a decrease in its incorporation may not permit sufficient numbers of protons to cross the membrane to allow dissociation of M1 from vRNPs. This would explain the decrease in infectivity of BM2ss1 virus in comparison to wild-type virus. Indeed, it appears that, unlike influenza A viruses (Watanabe et al., 2001), influenza B viruses absolutely require ion channel activity to be viable. Interestingly, complex formation by vRNP, M1, and NEP differs between influenza A and B viruses (Imai et al., 2003). Alternatively, BM2 may have another function within the virus replication cycle whose loss prevents the recovery of BM2 knockout virus. This study supports the hypothesis that BM2 is an ion channel required for virus disassembly (Paterson et al., 2003). Further work could include mutational analysis of the BM2 TM domain which should be key to the activity of the viral ion channel. It is noteworthy that this is the only region of the BM2 protein that is totally conserved among virtually all available BM2 sequences. However, definitive proof that BM2 is an ion channel whose activity is required for virus replication requires a drug that blocks the channel, which is as yet to be discovered.

Materials and methods

Cell culture and transfections

293-T cells and Madin–Darby canine kidney (MDCK) cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal calf serum, 1% L-glutamine (200 mM), 2% nonessential amino acids, and 1% penicillin–streptomycin (5000 IU/ml; 5000 µl) at 37 °C and 5% CO2. Transfections were performed on 6-well (immunofluorescence assay) or 12-well (Western blot assay and virus rescue) plates of 80% confluent 293-T cells in 3% DMEM. For five-plasmid transfections, 0.5 µg of each of pCIPB1, pCIPB2, pCIPA, and pPRM and 1 µg of pCINP were mixed with 140 µl serum-free DMEM and 10 µl FuGENE 6 transfection reagent (Roche) for 15 min at room temperature, added to cells, and incubated at 34 °C for 32 h.

Virus rescue and infections

Influenza B virus rescue was performed as described previously (Jackson et al., 2002). Briefly, 0.5 µg of pPRPB1, pPRPB2, pPRPA, pPRHA, pPRNP, pPRNA, pPRM, pPRNS, pCIPB1, pCIPB2, and pCIPA and 1 µg of pCINP were transfected into 80% confluent 293-T cells on a 12-well plate and incubated at 34 °C. Sixteen hours after transfection, the 293-T cells were co-cultured with MDCK cells in the presence of 2.5 µg of trypsin/ml. Cytopathic effect was observed 68–72 h post-transfection. For the production of pPRBM2stop and pPRBM2ss1 plasmids, site-directed mutagenesis (Stratagene) was performed using the pPRM plasmid as template.

For virus infections of MDCK cells, the virus was allowed to adsorb to cells at 34 °C for 1 h then removed and serum-free DMEM containing 2.5 µg of trypsin/ml was added followed by incubation at 34 °C. To concentrate virus, 40 ml of 1 × 107 pfu/ml virus was centrifuged at 25000 rpm through 30% sucrose for 2.5 h. Virus pellets were resuspended in 50 µl NTE buffer (150 mM NaCl; 10 mM Tris–HCl pH 7.5; 1 mM EDTA). Multistep growth curve infections were performed in 6-well plates of confluent MDCK cells using an MOI of 0.001 and supernatant samples were harvested every 12 h until 72 h postinfection. Single-step growth curve infections were performed in a similar way using an MOI of 5 and sampling every 3 h until 24 h postinfection. Samples were then used in plaque assay experiments.

Plaque assays and hemagglutination assays

Harvested supernatant samples were titrated in serial 10-fold dilutions in 200 µl volumes of serum-free DMEM on 12-well plates of confluent MDCK cells. After adsorption at 34 °C for 1 h, the virus was removed and cells were covered with overlay media (EMEM containing 7.5% BSA, 200 mM L-glutamine, 7.5% NaHCO3, 1 M HEPES, 1% dextran, 10000 units/ml penicillin, 10 mg/ml streptomycin, 0.6% oxoid agar) and incubated at 34 °C. Four days later, plaque number was counted and virus titers in pfu/ml were determined for each sample. For hemagglutination assays, virus samples were titrated in V-bottomed 96-well plates in serial 2-fold dilutions in 50 µl volumes of PBS. Fifty microliters of 0.5% chicken red blood cells was added and plates incubated on ice for 1 h. Virus titers in HA units/50 µl were determined for each sample.

SDS-PAGE and Western blotting

Confluent MDCK cells on 12-well plates were infected at an MOI of 1 and incubated at 34 °C for 16 h. Cells were lysed in 200 µl RIPA buffer (100 mM NaCl, 50 mM iodoacetamide, 1% Nonidet-P40, 0.1% SDS, 0.5% sodium deoxycholate, 20 mM Tris–HCl, pH 7.5) and proteins separated by SDS-PAGE on a 15% polyacrylamide gel. Proteins were transferred to a PVDF membrane and blocked overnight in 5% dried milk (Marvel) blocking buffer. Western blotting was performed using a mouse anti-NP monoclonal antibody (www.Immunologicalsdirect.com) at a 1/500 dilution in PBS–Marvel–0.1% Tween, a mouse anti-M1 monoclonal antibody (www.Immunologicalsdirect.com) at a 1/2000 dilution, or a rabbit anti-BM2 antibody at a 1/4000 dilution as the primary antibody. Membranes were washed in PBS–0.1% Tween followed by the addition of a
goat anti-mouse (1/10000 dilution) or goat anti-rabbit (1/4000 dilution) HRP-conjugated secondary antibody (www.Immunologicalsdirect.com). ECL Western blotting detection reagent (Amersham Biosciences) was added to each membrane according to the manufacturers instructions and membranes were developed on Hyperfilm ECL chemiluminescent film (Amersham Biosciences).

Immunohistochemical and immunofluorescence assays

For the immunohistochemical assay, MDCK cells were grown in 6-well plates, and for the immunofluorescence assay, MDCK cells were grown on 22-mm diameter glass coverslips in 6-well plates. Confluent MDCK cells were infected at a range of MOIs and incubated at 34 °C for 16 h. Cells were fixed in methanol/acetone (1:1) for 15 min and blocked in PBS–0.5% BSA–0.02% Na azide for 1 h at room temperature. The mouse anti-NP monoclonal antibody at a dilution of 1/100 was added to the cells for 1 h at room temperature. For the immunohistochemical assay, a mouse anti-human β-galactosidase-conjugated secondary antibody was added at a 1/100 dilution for 1 h at room temperature followed by the addition of β-gal substrate (0.5% X-gal, 10% CN buffer [30 mM K4Fe(CN)6, 30 mM K4Fe(CN)6, 10 mM MgCl2], in PBS) and incubated at 37 °C for 30 min. For the immunofluorescence assay, a goat anti-mouse FITC-conjugated secondary antibody was added to the cells at a dilution of 1/100 for 1 h at room temperature. Coverslips were then mounted on slides and viewed on a Leica confocal microscope. All antibodies used were obtained from www.Immunologicalsdirect.com.

To study the distribution of BM2 and HA within infected cells, confluent MDCK cells grown on glass coverslips in 6-well plates were infected with 5 × 10^5 pfu/ml Brec or BM2ss1 virus. Ten hours postinfection, cells were fixed in methanol/acetone (1:1) for 15 min, washed in PBS, and blocked in PBS–0.5% BSA–0.02% Na azide for 1 h at room temperature. A mouse anti-HA monoclonal antibody (Harlan Serolabs) at a 1/100 dilution or a rabbit anti-BM2 antibody at a 1/200 dilution was added to the cells for 1 h at room temperature. Coverslips were washed three times in PBS and either an anti-mouse or anti-rabbit FITC-conjugated secondary antibody at a 1/100 dilution or a rabbit anti-NB antibody at a 1/200 dilution in PBS–0.5% BSA–0.02% Na azide (PBN). Cells were incubated at room temperature for 1 h on a tube rotator. Cells were washed three times in PBN followed by centrifugation at 2000 rpm for 3 min. Cells were resuspended in 500 µl of either an anti-mouse or anti-rabbit FITC-conjugated secondary antibody at a 1/100 dilution in PBN and incubated at room temperature for 1 h on a tube rotator. Cells were washed three times following by centrifugation at 2000 rpm for 3 min. Cells were fixed in 500 µl CellFix (Becton Dickinson) and added to 1 ml of FACScanflow (Becton Dickinson). Samples were subjected to flow cytometry in a Becton Dickinson FACScan and results analyzed on WinMDI 2.8 software.

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References


