Phosphorylation of the M2 Protein of Influenza A Virus Is Not Essential for Virus Viability

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M2 is a minor component of the influenza A virus envelope. The cytoplasmic tail of the M2 protein is posttranslationally modified in the infected cell by palmitylation and phosphorylation. The primary site for phosphorylation of the M2 cytoplasmic tail is serine 64, which is highly conserved yet not required for the activity of the M2 ion channel. Using an exogenous incorporation assay, we have shown that incorporation of M2 into virus particles is type-specific and does not require phosphorylation of the cytoplasmic tail. In addition, phosphorylation of the cytoplasmic tail is not required for the directional transport of M2 in polarized MDCK cells. Using a reverse genetics and reassortment procedure, we generated a virus (Ra) specifically mutated in segment 7 such that the M2 cytoplasmic tail could no longer be phosphorylated. The virus was found to grow as well as wild-type virus in tissue culture and in eggs, was stable on passage in these systems, and possessed no second-site mutations in the engineered RNA segment. In vivo Ra replicated in Balb/c mice at least as well as the parent strain A/WSN/33. These studies indicate that phosphorylation of the M2 cytoplasmic tail is not required for in vitro or in vivo replication of influenza A virus. © 1998 Academic Press

INTRODUCTION

The M2 protein of influenza A virus is a small integral membrane protein and a minor component of the virion envelope (Lamb et al., 1985). M2 possesses an ion channel activity that is activated in the low pH environment of the endosome after the virus enters the cell (Hay et al., 1992; Pinto et al., 1992). The consequent acidification of the virion interior stimulates the dissociation of the internal virus components in preparation for replication (Martin and Helenius, 1991). Influenza B and C viruses possess similar small membrane proteins, termed NB and CM2, respectively (Betakova et al., 1996; Brassard et al., 1996; Hongo et al., 1997; Pekosz and Lamb, 1997), which may be functionally homologous to M2. However, these proteins share no sequence homology and are encoded by different strategies (Shaw and Choppin, 1984; Shih and Krug, 1996; Yamashita et al., 1988).

M2 is translated from a spliced message derived from RNA segment 7, which also encodes the matrix protein M1 (Lamb et al., 1981). The M2 protein has an amino-terminal ectodomain of 25 residues, which was recently shown to be important for incorporation of the protein into the influenza virus envelope (Park et al., 1998). The transmembrane domain of 19 hydrophobic residues possesses the ion channel activity that can be blocked by the antiviral agent amantadine (Duff et al., 1994). A function for the cytoplasmic tail of 54 amino acids has yet to be assigned, although the length and sequence of this region are well conserved among influenza A virus isolates (Fig. 1a), suggesting that an evolutionary pressure exists to maintain them. In particular, this region contains a number of serine residues, which serve as sites for phosphorylation, and a cysteine at residue 50, which receives fatty acid modification (see Fig. 1a). However, neither of these posttranslational modifications is required for the activity of the ion channel (Holsinger et al., 1995; Pinto et al., 1992). The extreme carboxyl-terminal four residues are completely conserved, and deletion of a single amino acid from this terminus results in virus attenuation. Furthermore, it has not been possible to recover viruses with five or 10 residues deleted from the C terminus (Castrucci and Kawaoka, 1995). In contrast, the cytoplasmic tails of the other two virion envelope proteins, haemagglutinin (HA) and neuraminidase (NA), can be deleted both individually or simultaneously without loss of virus viability (Garcia-Sastre and Palese, 1995; Jin et al., 1997). Incorporation of HA into influenza particles is not a type-specific event because influenza A virus HA proteins are incorporated into influenza B virus particles after double infection of cells (Sklyanskaya et al., 1985). Whether the NA proteins also show phenotypic mixing is not known, although it is noteworthy that an influenza A virus has been recovered in which the sequence of the cytoplasmic tail of NA was exchanged for that of an influenza B virus NA protein (Bilsel et al., 1993). The incorporation of M2 or NB proteins into heterotypic particles has not previously been investigated.

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During influenza A virus infection, high levels of M2 accumulate at the surface of infected cells, yet the protein is present at low frequency in the virus envelope (Zebedee and Lamb, 1988). This discrepancy implies that only a subset of M2 molecules within the cell are incorporated into particles. In the current study, we asked whether phosphorylation of the M2 cytoplasmic tail regulates incorporation of M2 molecules into particles and hence controls viral assembly. We tested whether unphosphorylated M2 protein would be incorporated into influenza virus particles in an exogenous incorporation assay. In parallel, we examined whether the incorporation of M2 is type-specific by asking whether M2 can be incorporated into influenza B virus particles. We have also ascertained whether the phosphorylation status of M2 affects its directional transport in polarized cells because phosphorylation of other proteins has been implicated in their targeting (Casanova et al., 1990; Jones et al., 1995).

Finally, bearing in mind the acute sensitivity of the virus to deletions of the M2 cytoplasmic tail and the conservation of the posttranslational modifications in this domain, we investigated whether a virus that contained only unphosphorylated M2 could be isolated using a reverse genetics procedure.

RESULTS

Incorporation of exogenous M2 proteins into virus particles

To detect incorporation of M2 protein into virus particles, we took advantage of the fact that the A/WSN/33 M2 migrates more slowly than M2 of A/PR/8/34 (Panayotov and Schlesinger, 1992) under nonreducing SDS–PAGE. We established a Madin–Darby canine kidney (MDCK) cell line that stably expresses the A/WSN/33 M2 protein. M2 expression at the cell surface was detected by immunohistochemical staining using the 14C2 monoclonal antibody as primary antibody. The levels of expression of M2 protein in these cells were similar to those found late in infection (Fig. 2a, lanes 6 and 7).

We next infected these cells with the A/PR/8/34 strain of influenza A virus at a high m.o.i. and analyzed the released virus for incorporation of exogenous M2 protein by Western blot, also using 14C2 (Fig. 2a). In virus released from cells expressing the exogenous M2 protein,

![Image of amino acid sequence alignment and cytoplasmic domain](attachment:alignment.png)
we could clearly detect both a slow and a fast migrating M2 species, indicating that the exogenous A/WSN/33 M2 protein had been incorporated (Fig. 2a, lane 2). When the same cells were infected with B/Lee/40, no M2 was detected in released virus (Fig. 2a, lane 4), although incorporation of the functionally homologous type B protein NB was readily seen (Fig. 2b, lanes 1 and 2). NB is visible as a single species in this experiment because the glycosylated species in released virus have been digested with endo-F and the B/Lee/40 infected cell lysate used as a control was generated in the presence of tunicamycin. These results demonstrate that incorporation of M2 protein into influenza virus particles is a type-specific event.

The primary site for phosphate addition on the A/Udorn/72 M2 cytoplasmic tail is at serine residue 64 (Holsinger et al., 1995). This residue, and the surrounding consensus for modification, is conserved in the M2 sequences accumulated to date (Fig. 1a). It has been demonstrated that modified M2 proteins which are unable to phosphorylate serine 64, use other serines for phosphorylation (Holsinger et al., 1995). The A/WSN/33 M2 protein contains two serines in the cytoplasmic domain at positions 64 and 71. To investigate the role of phosphate moieties on the M2 cytoplasmic tail, we established stable and clonal cell lines that expressed M2 proteins mutated (by serine-to-alanine substitutions) at serine 71 (P-2) and at both serines 64 and 71 (P-1–2) (Fig. 1b). It is interesting to note that we were not able to establish a cell line expressing M2 mutated at serine 64 alone (P-1), although we could detect expression of this protein after transient transfection (data not shown).

After infection of cells expressing either the P-2 or P-1–2 mutant M2 proteins, we monitored the M2 species in released virus by Western blotting. Only tetrameric
forms of M2 are shown in Fig. 3 because the small differences in migration between the M2 proteins are more easily seen with this analysis. Both singly phosphorylated and unphosphorylated forms of M2 were identified in released virions (Fig. 3, lanes 3 and 4). We also analyzed whether the mutant M2s were incorporated into B/Lee/40 particles, and confirmed that as for the wild-type protein, incorporation was type-specific (data not shown).

Transport of M2 in polarized epithelial cells

The phosphorylation state of some proteins controls directional transport in polarized epithelial cells (Casanova et al., 1990; Jones et al., 1995). Wild-type M2 is directed to the apical surface, where influenza virus budding occurs (Hughey et al., 1992). To determine whether phosphorylation plays a role in M2 targeting, MDCK cells expressing either the wild-type or mutant M2 proteins were cultivated on DATA filters to allow selective access to either the apical or basolateral surface. Surface expression of M2 was determined by staining either surface of the unfixed cells using 14C2. Staining was detected only when the apical membranes had received primary antibody, indicating that for both wild-type and mutant M2 proteins, transport was exclusively directed to the apical domain (Fig. 4). Thus phosphorylation of M2 does not influence directional transport and cannot account for the discrepancy in expression levels in the infected cell compared with the amount of M2 incorporated into budding particles.

Generation of an M2 phosphate-minus mutant by reverse genetics and analysis of phosphorylation of its proteins

We next asked whether there may be a role for M2 phosphorylation that would be apparent only in the context of the virus. We therefore attempted to rescue a transfecant virus in which the serine residues at both position 64 and 71 of A/WSN/33 M2 were replaced by alanine. The A/WSN/33 M2 protein is naturally resistant to the ion channel-blocking agent amantadine, and we took advantage of this observation in designing a rescue protocol. A synthetic RNA representing the mutated P-1±2 segment was transfected as RNP complexes into cells previously infected with an amantadine-sensitive virus, A/Eng/42/72. Progeny transfecant viruses were
selected in the presence of inhibitory concentrations of amantadine. A silent change that creates a unique SacI restriction enzyme site within codon 96 of the M2 message was introduced into the cDNA such that viruses derived from transfected RNAs could be identified by cleavage of their segment 7 RT-PCR products by SacI. In this way, a transfectant virus was identified in one of the four plaques picked after transfection of the phosphate-minus mutated RNA. Screening of the other three viruses showed that they did not contain the tagging mutation, and they are believed to be spontaneous escape mutants of the helper virus that have gained resistance to the antiviral agent (data not shown). The transfectant virus was plaque purified three times in the presence of amantadine and propagated in both MDCK cells and eggs. Viral RNA from purified virus grown in cells or eggs was used as template for RT-PCR of the entire segment 7 RNA. This product was cloned into pUC19 and sequenced. No changes were present in the RNA other than those that had been engineered (data not shown). The virus can grow to high titres in tissue culture and in eggs and was stable over at least six passages in these systems.

To confirm that the mutations introduced into the transfectant virus did abolish phosphorylation of M2, we conducted 32P-orthophosphate-labeling experiments of the phosphate-minus virus and labeled proteins from the helper A/Eng/42/72 and A/WSN/33 viruses as controls. M2 and NP proteins were immunoprecipitated from lysates of metabolically labeled infected cells. Fig. 5a shows that although M2 from both A/Eng/42/72 and A/WSN/33 viruses was labeled with 32P-orthophosphate (lanes 2 and 4), M2 from the transfectant phosphate-minus virus was not (lanes 1 and 3). A similar amount of M2 was labeled with radioactive cysteine and methionine for each virus (Fig. 5b, lanes 1-4), and orthophosphate labeling of NP was observed in all infected lysates (Fig. 5a, lanes 6-9).

Isolation of a reassortant virus lacking phosphorylation sites in M2 and growth in Madin-Darby bovine kidney cells

The transfectant virus described above contained 7 segments derived from A/Eng/42/72 (H3N2) with segment 7 derived from A/WSN/33 (H1N1). It has been previously observed that the M1 protein of H1 or H3 subtypes can affect growth of virus in tissue culture (Yasuda et al., 1994). Therefore, to assess whether there had been any subtle changes in phenotype as a result of the phosphate-minus mutation in M2 and to distinguish these from growth differences related to M1, we generated a reassortant virus Ra with the phosphate-minus mutations in segment 7 in an A/WSN/33 background.

The plaques produced by the Ra virus at 37°C were wild type in size and appearance. A multistep growth curve of the Ra virus was generated after infection at 37°C of Madin-Darby bovine kidney (MDBK) cells at an m.o.i. of 0.001 (Fig. 6). The virus showed no defect in replication in these permissive cells in comparison with the control virus A/WSN/33.

Growth of the Ra virus in vivo

To determine whether removal of the sites for phosphorylation of the M2 cytoplasmic tail affects the in vivo replication and virulence of influenza A virus, 6-week-old Balb/c mice were inoculated with Ra or the control strain A/WSN/33. The weight and disease status of individual mice were followed over an 7-day period. Both groups of infected mice lost weight compared with age-matched control animals at a similar rate (data not shown). Death occurred slightly earlier in the mice infected with Ra virus, and this difference was statistically significant by a
Multistep growth analysis of the Ra reassortant virus compared with wild-type A/WSN/33 virus in MDBK cells. MDBK cells were infected with reasortant (●) or wild-type virus (●) at an m.o.i. of 0.001. At various times after infection, cell supernatants were quantified by end point titration in MDBK cells.

Student's unpaired t test to a level of P < 0.005 (Table 1). All infected animals were dead by 7 days after inoculation with either virus.

On day 3 after virus administration, lungs were removed from five mice infected with each virus, and the amount of virus was quantified to assess the level of in vivo replication of each virus (Table 1). The titres of virus in the lungs of mice infected with Ra virus were ~0.5 log_{10}-fold higher than in lungs of mice infected with A/WSN/33 virus; again, this difference was significant (P < 0.005). Three plaques of Ra virus isolated from the lung of an infected mouse were amplified on MDBK cells, and the viral RNA was extracted. Sequencing of the M2 cytoplasmic tail region of segment 7 of these viruses revealed that there had been no reversion of the serine-to-alanine mutations, nor indeed were there any other changes of sequence in this region during in vivo replication (data not shown).

Taken together, these data indicate that the loss of phosphorylation on the cytoplasmic tail of M2 has not compromised the ability of the virus to replicate in the mouse lung.

**DISCUSSION**

Six of the influenza A virus proteins (NP, NS1, NS2, M1, PA, and M2) are reported to be phosphoproteins. The phosphorylation state of NP and M1 is known to control their intracellular distribution (Bui et al., 1996; Neumann et al., 1997). Phosphorylation and dephosphorylation events also control the direction of transport of cellular proteins such as the polymeric immunoglobulin receptor (plgR) and the protease furin in polarized epithelial cells (Casanova et al., 1990; Jones et al., 1995). Influenza viruses bud exclusively from the apical membrane of polarized cells, and it is known that M2, a structural component of the virus, is transported predominately to the apical side (Hughey et al., 1992). In this study, we investigated the transport of M2 phosphorylation mutants in polarized epithelial cells. By preventing phosphorylation at serine 71 or at both serines 64 and 71, no differences in cell surface expression were seen (Fig. 4). Furthermore, both the wild-type and mutant proteins were transported to the cell surface at the same rate (data not shown). We conclude that the phosphorylation state of the cytoplasmic tail of M2 does not control its intracellular transport.

Cytoplasmic tail phosphorylation on some proteins can control their interactions with cytosolic components. In the context of the influenza A virus M2 protein, such interactions might be involved in viral assembly, possibly through binding to the matrix protein M1. Earlier genetic evidence had suggested a functional relationship between M2 and M1. The anti-M2 monoclonal antibody 14C2 was shown to inhibit virus budding (Hughey et al., 1995). Furthermore, virus mutants that escape inhibition by this M2-specific antibody have sequence changes in either the M2 ectodomain (the antibody epitope), the M2 cytoplasmic tail, or M1 (Zebedee and Lamb, 1989). Interestingly, one of the changes seen in the M2 cytoplasmic tail was a mutation of the serine at position 71, a site in the correct consensus for phosphorylation. It has also been shown that the escape mutants with sequence changes in M1 display a different morphology, suggesting that the M1/M2 relationship might control virion morphology (Roberts et al., 1998). On the other hand, in two separate studies, coexpression of M2 (or HA or NA) did not increase the proportion of M1 that associates with membranes (Kretzschmar et al., 1996; Zhang and Lamb, 1996), whereas a third report showed that HA or NA could enhance M1 membrane association (Enami and Enami, 1996). The latter study did not assess the effect of M2 coexpression.

We hypothesized that phosphorylation might control

**TABLE 1**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titre in the lung as TCID_{50}/lung (number of animals)</th>
<th>Time to death as h p.i. (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td>5.30 ± 0.22 (n = 5)</td>
<td>142.3 ± 28.1 (n = 14)</td>
</tr>
<tr>
<td>Ra</td>
<td>5.81 ± 0.19 (n = 5)</td>
<td>117.0 ± 16.7 (n = 16)</td>
</tr>
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Note. Six week-old male Balb/c mice were infected intranasally with 25 µl of the A/WSN/33 virus or Ra virus containing 7.5 × 10^{6} pfu. Virus titres in the lungs were determined 3 days after infection by assaying infectivity in MDBK cells. Values shown are mean ± SD. Statistical significance for the differences between A/WSN/33 and Ra effects was calculated using a Student's unpaired t test. For lung titres, this value was P < 0.005, and for time to death, the value also was P < 0.005.
incorporation of M2 into virus particles. However, we show here that unphosphorylated M2 is efficiently incorporated into virus particles (Fig. 3, compare band a in lane 2 with band b in lane 4). Furthermore, it is unlikely that unphosphorylated M2 is incorporated by forming hetero-oligomers with a phosphorylated species provided by the infecting A/PR/8/34 virus because we were able to recover a transfected virus whose M2 cannot be phosphorylated. The amount of M2 incorporated into the envelope of this recombinant virus is different from that for wild-type virus (data not shown). This result confirms that phosphate is not required for M2 incorporation. It remains a possibility that phosphorylation actually prevents M2 incorporation. Indeed, some other enveloped viruses use a dephosphorylation event at the plasma membrane to trigger the interaction of core components with envelope proteins, enabling envelopment and budding to occur. The E2 protein of Sindbis virus requires phosphorylation to release its cytoplasmic tail from the plasma membrane followed by a dephosphorylation event, which allows the cytoplasmic tail to interact with the nucleocapsid and assembly to occur (Liu and Brown, 1993). In the case of duck hepatitis B virus, assembly is believed to be a result of dephosphorylation of the core protein because phosphorylated core protein could not be detected in purified virions (Pugh et al., 1989). To our knowledge, it has not been previously demonstrated that the phosphorylated species of M2 is incorporated into particles. Our preliminary experiments demonstrated that 32P-orthophosphate-labeled M2 is associated with purified virions (data not shown). The issue of how the levels of incorporation of M2 into the envelope are controlled remains unresolved. Interestingly, it was recently demonstrated that the HA transmembrane domain sequence determines its transport to specific areas of the cell membrane termed sphingolipid rafts, which differ in lipid composition from other plasma membrane regions (Scheiffele et al., 1997). Presumably, these are the sites of influenza virus assembly and budding. It may be that M2 is not delivered efficiently to these areas and thus not physically available for efficient incorporation into particles along with HA and NA. This possibility is currently under investigation. Regardless of the mechanism, the virus has evolved to limit the amount of M2 allowed in the particle for a reason. It is tempting to speculate that too many ion channels might overacidify the virion interior with deleterious effects on the internal proteins and the uncoating mechanism.

We demonstrated in this study that unlike the situation for the other influenza A virus envelope proteins, HA and NA, M2 incorporation into virions is type-specific. The NB protein of influenza B virus is thought to be the functional homolog of M2, although it is not phosphorylated (Brassard and Lamb, 1996). Therefore, we tested whether an unphosphorylated M2 protein would be incorporated into influenza B virus envelopes. However, this was not the case, suggesting that it is not the phosphate moiety but specific sequences in M2 that direct the type-specific incorporation. Indeed, a recent study by Park et al. (1998) demonstrated that the M2 ectodomain is required for incorporation of a chimeric protein into influenza A virus particles. These authors hypothesized that this region of M2 might interact with the HA ectodomain or transmembrane domain. To be compatible with the present data, this model would require that M2 does not interact with the equivalent sequences of influenza B virus HA.

In addition, we used a reverse genetics procedure to generate a mutant virus that is unable to phosphorylate the M2 cytoplasmic tail. Neither the transfected virus nor a reassortant virus (Ra) with the same mutated RNA segment, could phosphorylate M2. In addition, the phosphate-minus viruses showed little phenotypic difference in MDCK cells, MDBK cells, or eggs compared with control viruses. It is likely that the pressures exerted on viruses replicating in the whole host organism are different from those in the highly permissive in vitro systems. Indeed, the conservation of phosphorylation sites within the M2 sequence over time suggests that there is an advantage conferred by possessing phosphorylated M2 that has not been detected here. Other negative-strand RNA viruses encode viral proteins or possess protein modifications that are not essential for in vivo viability (Bukreyev et al., 1997; Radecke and Billeter, 1996; Sakaguchi et al., 1997). In some of these cases, the mutation has subsequently been shown to attenuate virus pathogenesis in vivo (Bukreyev et al., 1997; Kato et al., 1997). In other situations, there was an enhancement of replication or pathogenicity in mice. For example, an influenza A virus lacking cysteine residues in M2 replicated to higher titres in the mouse upper respiratory tract (Castrucci et al., 1997), and an engineered VSV in which the gene order was rearranged killed mice at a lower dose than the wild type (Wertz et al., 1997). It is conceivable that viruses that kill more slowly have a subtle evolutionary advantage over more pathogenic variants because the potential for transmission might be increased. Our studies have shown that loss of phosphorylation on M2 has not compromised the replication of influenza virus in vivo. Indeed, higher viral titres were recovered from lungs of mice infected with Ra than from those infected with the control virus A/WSN/33. Furthermore, the mice infected with equivalent amounts of virus were killed more quickly by the virus in which the M2 protein was not phosphorylated. In future work, we intend to determine the basis for this difference.

METHODS AND MATERIALS

Viruses and cells

Influenza viruses A/WSN/33, A/PR/8/34, A/Eng/42/72, and B/Lee/40 were cultivated in 10-day-old embryonated chicken eggs at 37°C or 34°C for influenza type A or B
viruses, respectively. Allantiotic fluids were harvested 2 days after inoculation. Virus stocks were titered on MDCK cells and stored at −70°C. MDCK and MDBK cells were routinely passaged in Eagle’s minimal essential medium (EMEM) (GIBCO BRL) supplemented with 10% heat-inactivated FCS (GIBCO BRL).

**DNA cloning**

Segment 7 of A/WSN/33 was cloned as a cDNA between the EcoRI and XbaI restriction sites of the multiple cloning site of pUC19, after RT-PCR from vRNA using primers EKFlu (5'-gcgcaatctccttgAGACAAAGACG-3') and AllfluPCR (5'-aagctctagaattccctactaaATGAGAACAAGGTAGTTTTTACTCGAGCTTATG-3'); lowercase letters denote noninfluenza sequences included for cloning purposes), giving the plasmid pT3WSNMut1. In vitro transcription of Ksp632I linearized pT3WSNMut1 by T3 polymerase produces an RNA with an authentic segment 7 terminus and a silent mutation at nucleotide 96 (G→C), which creates an SacI restriction enzyme site as a tag. To create cDNAs encoding M2 proteins with mutations of serine to alanine at amino acids 64 and 71, or 64 or 71 alone, PCR mutagenesis was performed on template plasmids containing one or both mutations were named pT3WSNMP-1, pT3WSNMP-2, and pT3WSNMP-1+2.

Cell lines for the expression of A/WSN/33 M2 and the mutant proteins P-2 and P-1+2

The plasmid pT3WSNMMut1 or plasmids containing the mutations encoding serine-to-alanine changes described above were used as template for PCR with primers M2START (5'-cccgggatctAGATGCTTATTTACTACGAAACCGCCTTATCAGAAAGGAAGGGGTCGC-3') and M2STOP (5'-cccggggatctCTAATCCACGCTTATG-3'), generating cDNAs corresponding to the M2 spliced mRNAs. These products were subcloned into the mammalian expression vector pEE6.hcmv.Neo (Bebbington, 1991) between the XbaI and EcoRI restriction enzyme sites downstream of the hCMV promoter.

MDCK cells were transfected using Pfx-2 lipofection reagent (InVitrogen) with 2 μg of pEE6.Neo.M2, pEE6.Neo.P-1M2, pEE6.Neo.P-2M2, or pEE6.Neo.P-1-2M2 encoding the wild-type M2 or the phosphorylation mutants P-1, P-2, and P-1-2 (Fig. 1b). Stably expressing colonies were selected in EMEM supplemented with 10% FCS and 500 μg/ml geneticin sulfate (GIBCO BRL). Expressing colonies were screened by blue cell assay (Ward et al., 1994) using an anti-M2 monoclonal antibody, 14C2 (kindly supplied by R. A. Lamb), diluted in PBS plus 0.5% bovine serum albumin (BSA) and 0.02% sodium azide as a primary antibody and a β-galactosidase-conjugated anti-mouse secondary antibody (Harlan Sera-lab), followed by incubation with X-gal substrate. Colonies expressing exogenous M2 at levels comparable to those seen in infected cells were amplified and maintained in selective media.

**Preparation of whole cell lysates and infected cell lysates**

For whole cell lysates, MDCK and MDCK cells expressing the wild-type and mutant M2s were grown to confluence in 35-mm dishes. Cells were washed in ice-cold PBS and incubated with lysis buffer (100 mM NaCl, 50 mM iodoacetamide, 1% Nonidet-P40, 0.1% SDS, 0.5% sodium deoxycholate, 20 mM Tris·HCl, pH 7.5) on ice for 10 min.

For infected cell lysates, MDCK cells were infected at an m.o.i. of 3 with A/PR/8/34 or B/Lee/40 and incubated for 12 h at 37°C or 34°C, respectively. Lysates were stored at −20°C. B/Lee/40 was amplified in the presence of 2.5 μg/ml tunicamycin (Sigma).

**Generation and purification of virus incorporating exogenous M2 proteins**

MDCK cells expressing wild type or the mutant M2s were infected with A/PR/8/34 or B/Lee/40 at an m.o.i. of 3. Virus was allowed to adsorb for 1 h at the appropriate temperature. Cells were washed twice in PBS and incubated in serum-free EMEM including 2 μg/ml trypsin (Worthington Biochemical). After incubation at the appropriate temperature for 48 h, supernatants containing released virus were harvested. Cell debris was removed by centrifugation at 10,500g in a benchtop microfuge for 15 min. Virus was pelleted from the supernatants through cushions of 30% sucrose in NTE (100 mM NaCl, 10 mM Tris·HCl, pH 7.4, 1 mM EDTA) by ultracentrifugation at 45,000 rpm using an SW55 rotor (Beckman) at 4°C for 90 min. Virus was resuspended in TMK (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris·HCl, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and 2 μg/ml aprotinin (Sigma) and stored at −20°C until analysis.

**Analysis of virus to detect the incorporation of exogenous M2**

Purified virus and cell lysates were analyzed by SDS-PAGE using 15% or 20% acrylamide gels under nonreducing or reducing conditions for detection of M2 and NB, respectively. Proteins were transferred onto PVDF membranes (Immobion P; Millipore) and probed either for M2, with 14C2 diluted in 2% Marvel milk protein in PBS containing 0.1% Tween 20, or for NB, using a rabbit polyclonal antiserum generated to a histidine-tagged cytoplasmic tail fragment of NB expressed in Escherichia coli. Primary antibodies bound
to M2 or NB were detected with anti-mouse or anti-rabbit HRPO-conjugated secondary antibodies (Amersham), respectively. Proteins were visualized using an ECL detection system (Amersham) and exposure to photographic film.

Cell surface expression of M2 and mutant M2 proteins in polarized epithelial cells

MDCK cells stably expressing the wild-type M2 and the phosphorylation mutants were grown to confluency on DATA filters (0.4-μm pore size; Costar). Blue cell assay was performed on unfixed, nonpermeabilized cells. The cells were probed on either the apical or basolateral surface for expression of the M2 proteins using 14C2 as a primary antibody, followed by a secondary anti-mouse antibody conjugated to β-galactosidase (Harlan Sera-lab). Finally, cells were incubated in X-Gal substrate at 37°C (Ward et al., 1994).

Generation and identification of transfectant viruses

RNP transfections were performed in MDBK cells essentially as described previously (Enami et al., 1990; Enami and Palese, 1991). A/Eng/42/72 was selected from a panel of potential H3N2 helper viruses because it showed clear inhibition of plaque formation in the presence of 100 mM amantadine HCl (Sigma). The transfected segment 7 is derived from the A/WSN/33 strain, which encodes an M2 ion channel that is naturally amantadine resistant. After three rounds of plaque purification of potential transfectant viruses in the presence of amantadine, individual plaques were amplified in MDCK cells to produce virus stocks. Viral RNA was extracted from 3 ml of tissue culture supernatant as described previously (Stevens and Barclay, 1998). Viral RNA was subjected to RT-PCR using primer M2RT (5′-GGCTATGGAGCAATGTGGTTG-3′) complementary to nucleotides 587–607 of segment 7 for RT and primers M2seq (5′-CCTATCATGAACGATGGGGG-3′) complementary to nucleotides 742±72 with Allflupcr for PCR. The product of 315 bp was digested with SacI, and cleavage into two fragments of 58 and 257 bp confirmed the presence of the restriction site tag and that the vRNA was derived from a transfectant virus.

32P-Orthophosphate and 35S-methionine and -cysteine labeling and immunoprecipitation of virus

MDCK cells were incubated overnight in phosphate-free EMEM and then infected with virus at an m.o.i. of >3. At 5 h p.i., 5 mCi of 32P-orthophosphate was added to the medium, and incubation continued for an additional 3 h. For methionine and cysteine labeling, at 4 h p.i., duplicate dishes were incubated in methionine- and cysteine-free media (Sigma) for 1 h. Then, 10 μCi of 35S-methionine and -cysteine was added, and incubation was continued for an additional 3 h.

At 8 h p.i., cells were washed with either phosphate-free EMEM or PBS for orthophosphate or methionine-cysteine labeling, respectively, and lysed in a total volume of 500 μl of lysis buffer. Cell lysates were passed three times through a fine-gauge syringe, and nuclei and membranes were pelleted by centrifugation at 80,000 rpm in a Tw2 rotor of a Beckman benchtop ultracentrifuge for 30 min at 4°C. Next, 200 μl of clarified cytoplasmic extracts were incubated at 4°C overnight with either 2 μl of 14C2 or 5 μl of anti-NP monoclonal antibody (Harlan Sera-lab). Protein G–agarose (30 μl) previously washed in lysis buffer was added, and tubes were rotated for 30 min at room temperature. Agarose beads, to which antibodies and antigens were bound, were washed five times with lysis buffer and resuspended in 30 μl of Laemmli’s buffer containing the reducing agent β-mercaptoethanol. Immune precipitates were analyzed by 15% SDS±PAGE. Gels were treated with Amplify (Amersham) before drying and exposure to β-max hyperfilm (Amersham).

Generation of the reassortant virus Ra lacking phosphorylation sites in M2

A virus with seven RNA segments from A/WSN/33 and segment 7 containing the engineered mutation was generated by reassorting the transfectant virus with ts51 (obtained from Dr. Gary Whittaker, Cornell University). Ts51 is a derivative of A/WSN/33 that contains a point mutation in the M1 gene, rendering the virus temperature sensitive (Whittaker et al., 1995). The transfectant virus was exposed to UV irradiation (wavelength 220 nm) for 20 min at a distance of 10 cm to destroy infectivity and, in particular, target the large RNA segments. This preparation was used to infect MDCK cells that had previously been infected with a high m.o.i. of ts51 virus. After overnight incubation at 39°C, the supernatants were plated in MDBK cells. Plaques were picked and amplified and then screened by RT-PCR for the presence of the SacI tag and by analysis of protein migration by SDS±PAGE to confirm that only segment 7 had been transferred (data not shown).

Growth of the Ra virus in MDBK cells

MDBK cells were infected with the Ra virus and A/WSN/33 control virus at an m.o.i. of 0.001 and incubated at 37°C. Culture supernatants were harvested at various times after infection, and the titer of released virus was established by plaque assay in MDBK cells.

Growth of the Ra virus in vivo

Six-week-old Balb/c mice were inoculated intranasally under halothane anaesthesia with 7.5 × 105 pfu of the Ra virus or A/WSN/33 in a volume of 25 μl. The weight and disease status of individual mice were monitored over an 7-day period. On day 3 after virus administration, five
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