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**Title**
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Influenza A Virus Can Undergo Multiple Cycles of Replication without M2 Ion Channel Activity

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Ion channel proteins are common constituents of cells and have even been identified in some viruses. For example, the M2 protein of influenza A virus has proton ion channel activity that is thought to play an important role in viral replication. Because direct support for this function is lacking, we attempted to generate viruses with defective M2 ion channel activity. Unexpectedly, mutants with apparent loss of M2 ion channel activity by an in vitro assay replicated as efficiently as the wild-type virus in cell culture. We also generated a chimeric mutant containing an M2 protein whose transmembrane domain was replaced with that from the hemagglutinin glycoprotein. This virus replicated reasonably well in cell culture but showed no growth in mice. Finally, a mutant lacking both the transmembrane and cytoplasmic domains of M2 protein grew poorly in cell culture and showed no growth in mice. Thus, influenza A virus can undergo multiple cycles of replication without the M2 transmembrane domain responsible for ion channel activity, although this activity promotes efficient viral replication.

Cell membranes consist of a double layer of lipid molecules in which various proteins are embedded. Because of its hydrophobic interior, the lipid bilayer of a cell membrane serves as a barrier to the passage of most polar molecules and therefore is crucial to cell viability. To facilitate the transport of small water-soluble molecules into and out of cells and intracellular compartments, such membranes possess carrier and channel proteins. Ion channels are essential for many cellular functions, including the electrical excitability of muscle cells and electrical signaling in the nervous system (1). They are present not only in all animal and plant cells and microorganisms, but have also been identified in viruses (12, 31, 32, 33, 37, 43, 44, 45), in which they are thought to play an important role in replication.

The influenza A virus is an enveloped negative-strand virus with eight RNA segments encapsidated with nucleoprotein (NP) (24). The viral membrane is three proteins: hemagglutinin (HA), neuraminidase (NA), and M2. The life cycle of viruses generally involves attachment to cell surface receptors, entry into the cell, and uncoating of the viral nucleic acid, followed by replication of the viral genes inside the cell. After the synthesis of new copies of viral proteins and genes, these components assemble into progeny virus particles, which then exit the cell (34). Different viral proteins participate in each of these steps. In influenza A viruses, the M2 protein, which possesses ion channel activity (32, 43, 44), is thought to function at an early stage in the viral life cycle, between host cell penetration and uncoating of viral RNA (16, 26, 44). Once virions have undergone endocytosis, the virion-associated M2 ion channel is believed to permit protons to flow from the endosome into the virion interior to disrupt acid-labile M1 protein-ribonucleoprotein complex (RNP) interactions, thereby promoting RNP release into the cytoplasm (16). In addition, among some influenza virus strains whose HAs are cleaved intracellularly (e.g., A/fowl plaque/Rostock/34 [FPV Rostock]), M2 ion channel activity is thought to raise the pH of the trans-Golgi network, preventing conformational changes in the HA due to conditions of low pH in this compartment (15, 29, 46).

Evidence that the M2 protein has ion channel activity was acquired by expressing the protein in oocytes of Xenopus laevis and measuring membrane currents (18, 32, 49). Specific changes in the M2 protein transmembrane (TM) domain altered the kinetics and ion selectivity of the channel, providing strong evidence that the M2 TM domain constitutes the pore of the ion channel (18). In fact, the M2 TM domain itself can function as an ion channel (10). Because a requirement for M2 ion channel activity in the replication of influenza A viruses has not been directly established, we generated a series of viruses with defective M2 ion channel activity using a recently established reverse-genetics system (13, 27) and tested their replication in cell culture and mice.

MATERIALS AND METHODS

Cells and viruses. 293T human embryonic kidney cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and in minimal essential medium (MEM) containing 5% newborn calf serum, respectively. The 293T cell line is a derivative of the 293 line into which the gene for the simian virus 40 T antigen was inserted (9). All cells were maintained at 37°C in 5% CO2.

A/Udorn/307/72 (H3N2) virus was propagated in 10-day-old embryonated chicken eggs. Construction of plasmids. The cDNA of Udorn virus was synthesized by reverse transcription of viral RNA with an oligonucleotide complementary to the conserved 3' end of viral RNA, as described by Katz et al. (21). The cDNA was amplified by PCR with M gene-specific oligonucleotide primers containing BamHI sites, and PCR products were cloned into the pT7Blueblunt vector (Novagen, Madison, Wis.). The resulting construct was designated pTPolIUdM.
Replicative properties of transfectant viruses. Transfectant viruses were grown in MDCK cells containing 0.5 \( \mu \)g of trypsin per ml and purified by centrifugation through six-step sucrose gradients (20, 30, 35, 40, 45, and 50%) for 2.5 h at 50,000 \( \times \) g at 4°C. Fractions (0.3 ml each) were then collected through a hole pierced in the bottom of the tube and assayed by hemagglutination for the presence of virus. The fractions that contained virus were pooled and spun down at 100,000 \( \times \) g for 1 h at 4°C, resuspended in phosphate-buffered saline (PBS), and stored in aliquots at −80°C. Purified virus was resuspended in lysis buffer (0.6 M KCl, 50 mM Tris-HCl [pH 7.5], 0.5% Triton X-100). The viral lysates were plated on sodium dodecyl sulfate (SDS)–15% polyacrylamide gels, which were electrophoresed at 4°C with 5% skim milk in PBS and incubated with the 14C2 anti-M2 monoclonal antibody (kindly provided by R. Lamb) and anti-WSN-NP monoclonal antibody for 1 h at room temperature. The membrane was washed three times with PBS containing 0.05% Tween 20. Bound antibodies were detected with a Vectastain ABC kit (Vector) and the Western immunoblot ECL system (Amersham). Signal intensities were quantified with an Alpha Imager 2000 (Alpha Innotech Corporation).

**RESULTS**

Generation of influenza A viruses containing mutations in M2 protein. The TM domain of the M2 protein possesses an \( \alpha \)-helical structure (10, 35, 44). Mutations at residues V-27, A-30, S-31, G-34, and L-38, all of which are located on the same face of the \( \alpha \)-helix, alter the properties of the M2 ion channel (14, 32, 49). To determine the role of the ion channel activity of M2 in viral replication, we initially constructed four plasmids and used them to generate mutant viruses with changes in the M2 TM domain (Fig. 1). The whole-cell currents of the mutant proteins, expressed in oocytes of *Xenopus laevis*, were measured by Holsinger et al. (18), using a two-electrode voltage clamp procedure. Two mutants, M2A30P and M2del29-31, no detectable ion channel activity; +, detectable ion channel activity.
and M2del29-31, had no functional ion channel activity at either neutral or low pH. M2V27T and M2S31N, which showed ion channel activity at low pH (18), were used as positive controls.

To generate mutant viruses by plasmid-driven reverse genetics (27), we transfected 293T cells with nine protein expression plasmids and eight that directed the production of rRNA segments encoding all WSN viral genes except the M gene, which was derived from Udorn virus (wild type). The corresponding transfectant viruses were designated M2V27T, M2A30P, M2S31N, M2del29-31, and WSN-UdM (for the virus containing the wild-type Udorn M gene).

To determine the efficiency of virus generation, we titrated viruses in the culture supernatant of 293T cells at 48 h posttransfection with MDCK cells. As shown in Table 1, more than 10^7 transfectant viruses with the wild-type or mutant M gene were present. Thus, all viruses bearing M2 mutations and the virus possessing the wild-type Udorn M gene were generated with similar efficiencies. The transfectant viruses were plaque purified once in MDCK cells and then inoculated into MDCK cells to make virus stocks. The stability of the introduced mutations was analyzed by sequencing the M gene segments of the transfectant viruses after 10 passages in MDCK cells. No revertants were found (data not shown).

**Growth properties of M2 mutant viruses in tissue culture.**

We next compared the growth properties of M2 ion channel mutants and wild-type WSN-UdM virus in MDCK cells (Fig. 2). Cells were infected at an MOI of 0.001, and yields of virus in the culture supernatant were determined at different times postinfection at 37°C. The mutant viruses did not differ appreciably from the wild-type WSN-UdM virus in either growth rate (Fig. 2) or the size of plaques after 48 h of growth (1.5 mm in diameter).

To assess the amantadine sensitivity of these viruses, the M2 mutant and wild-type WSN-UdM viruses were grown in MDCK cells in the presence of different concentrations of amantadine. In cell culture, amantadine produces two discrete concentration-dependent inhibitory actions against viral replication. A nonspecific action at concentrations of >50 μM, resulting from an increase in the pH of endosomes, inhibits activation of HA membrane fusion activity involved in endocytosis (7), whereas at lower concentrations, 0.1 to 5 μM, the drug selectively inhibits viral replication (2). As shown in Fig. 3, amantadine markedly reduced the yield of wild-type WSN-UdM virus as well as the size of plaques (data not shown) at each of the three test concentrations. By contrast, at 5 μM amantadine, the replication of M2 mutant viruses was either not affected or inhibited only slightly. Substantial inhibition due to the drug’s nonspecific activity was seen at 50 μM. Thus, all of our M2 mutants were more resistant to amantadine than the wild-type virus.

**Generation of transfectant viruses in which M2 TM domain was replaced with that from HA or NA.** Although the M2A30P and M2del29-31 mutants do not have functional ion channel activity (which was shown by Holsinger et al. [18] using a

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**TABLE 1. Virus titers in the supernatant of 293T cells after plasmid transfection**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.9 × 10^5</td>
</tr>
<tr>
<td>M2V27T</td>
<td>6.0 × 10^6</td>
</tr>
<tr>
<td>M2A30P</td>
<td>1.1 × 10^6</td>
</tr>
<tr>
<td>M2S31N</td>
<td>1.2 × 10^6</td>
</tr>
<tr>
<td>M2del29-31</td>
<td>1.7 × 10^6</td>
</tr>
<tr>
<td>M2HATM</td>
<td>2.2 × 10^6</td>
</tr>
<tr>
<td>M2NATM</td>
<td>2.2 × 10^6</td>
</tr>
<tr>
<td>D2TMVCT</td>
<td>1.4 × 10^6</td>
</tr>
</tbody>
</table>

*293T cells were transfected with eight plasmids for the production of A/WSN/33 vRNA (excluding the M gene, which was derived from A/Udorn/72 virus) and nine protein expression plasmids, as described in Materials and Methods. At 48 h posttransfection, we titrated virus in the supernatant of 293T cell cultures using MDCK cells.*

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**FIG. 2. Growth curves of M2 mutant and wild-type WSN-UdM viruses.** MDCK cells were infected with virus at an MOI of 0.001. At the indicated times after infection, the virus titer in the supernatant was determined. The values are means of triplicate experiments. The standard deviation (SD) is less than 0.59 for each sample. □, M2V27T; ■, M2A30P; △, M2S31N; ▲, M2del29-31; ○, wild type.

**FIG. 3. Amantadine sensitivity of M2 ion channel mutants.** The mutant and wild-type WSN-UdM viruses were tested for plaque-forming capacity in MDCK cells in the presence of different concentrations of amantadine. Experiments were performed three times, with the results reported as means ± SD. ■, M2V27T; □, M2A30P; ●, M2S31N; △, M2del29-31; ▲, wild type.
two-electrode voltage clamp procedure), they both replicated as well as the wild-type virus in MDCK cells (Fig. 2). However, we could not rule out the possibility of low-level ion channel activity below the sensitivity range of the assay. For this reason, we attempted to generate chimeric mutant viruses in which the M2 TM domain was replaced with that from the HA or NA of the A/WSN/33 virus (Fig. 4). When we assayed the supernatant of 293T cells transfected with plasmids for virus production, the chimeric mutants M2HATM and M2NATM were each viable, but their titers were more than 10-fold lower than that of the wild-type WSN-UdM titer (Table 1). The mutants also produced small plaques (1.0 mm in diameter) after 48 h of growth. Thus, influenza A virus can replicate without the M2 TM domain in cell culture.

Generation of transfectant ΔM2TMCYT virus lacking M2 TM and cytoplasmic domains. Although the M2HATM and M2NATM viruses lack the M2 TM domain, their M2 proteins are membrane anchored. Thus, we conducted a more rigorous test of the requirement for M2 ion channel activity in influenza A virus replication. By constructing a mutant M gene possessing two stop codons at the 3’ end of the M1 open reading frame (ORF), we attempted to produce a mutant virus with an M gene that encodes intact M1 protein and a truncated M2 corresponding to the ectodomain (23 amino acids), but lacking both a TM domain and a cytoplasmic tail (Fig. 4). The resultant virus, ΔM2TMCYT, was viable (titer of 1.4 × 10^4 PFU per ml of supernatant from 293T cell cultures transfected with plasmids for virus production [Table 1]) and produced pinpoint plaques (~0.5 mm in diameter). The titer of the stock virus was 1 × 10^8 PFU per ml.

Growth properties of M2HATM and ΔM2TMCYT viruses in cell culture. MDCK cells were infected with M2HATM at an MOI of 0.001 PFU per cell and with ΔM2TMCYT at an MOI of 0.01 PFU per cell and incubated at 37°C. Although M2HATM produced a lower titer than the wild-type WSN-UdM virus at 12 and 24 h postinfection, its maximum titer at 36 h was almost the same as that of the wild-type virus (Fig. 5). By contrast, ΔM2TMCYT grew very slowly, reaching its maximum titer at 108 h postinfection (Fig. 6A). Interestingly, at 33°C, this mutant attained a titer of nearly 10^6 PFU per ml, equivalent to that of the wild-type virus (Fig. 6B), although its growth was substantially slower. These results indicate that influenza A virus can undergo multiple cycles of replication without the M2 TM and cytoplasmic domains, although these domains are both important for efficient viral replication.

Incorporation of mutant M2 molecules into virions. Conceivably, the M2 point and chimeric mutants possessed some residual ion channel activity, so that increased incorporation of the M2 protein into virions could compensate for any defect in this function. We therefore compared the efficiency of incorporation of the wild-type and mutant M2s into influenza virions by Western blot analysis after standardization based on the intensity of NP expression (Fig. 7). Virion incorporation of M2del29-31 and M2HATM M2 proteins was slightly reduced compared with the wild-type protein. The band detected slightly below the M2 protein of the wild-type virus is probably a proteolytically cleaved form of M2, as reported by others (51). An additional band below the NP protein, which was reactive with anti-NP but not anti-M2 antibody, is a cleavage product of NP (53). Together, these results demonstrate that increased incorporation of M2 protein into virions probably does not compensate for defective M2 ion channel activity.

Kinetics of viral protein synthesis in mutant and wild-type virus-infected cells. To determine whether the lack of M2 ion channel activity, as detected with the in vitro assay, affects the kinetics of viral replication, we examined the kinetics of viral protein production in MDCK cells that were infected with mutant or wild-type viruses. Similar results were obtained for the A30P, del29-31, HATM, and wild-type WSN-UdM viruses at 2, 4, 6, and 8 h postinfection (data not shown).

Replication of M2 mutant viruses in mice. To validate our in vitro test results in an animal model, we infected mice with each of our six mutant viruses (Table 2). M2A30P virus repli-
cated in the lungs as well as the wild-type WSN-UdM and control M2V27T and M2S31N mutants, while replication of M2del29-31 virus in this organ was more than 10-fold lower. By contrast, neither the M2A30P nor the M2del29-31 virus was found in nasal turbinates from any of the infected mice. M2HATM and ΔM2TMCYT viruses were not recovered from either the lungs or the nasal turbinates. These results establish that M2 ion channel activity is necessary for efficient influenza A virus replication in vivo.

**DISCUSSION**

We used a new reverse-genetics system (27) to generate transfectant influenza A viruses with changes in the M2 TM domain sufficient to block ion channel activity according to in vitro assays (18). Despite this functional defect, all of the mutant viruses replicated as efficiently as the wild-type WSN-UdM virus in cell culture, although we could not rule out the possibility of residual ion channel activity adequate to support viral replication. Experiments in which the TM domain of the M2 protein was replaced with that from the HA (M2HATM) or NA (M2NATM) or was completely deleted together with the cytoplasmic domain (ΔM2TMCYT) demonstrated that influenza A virus can undergo multiple cycles of replication in cell culture without M2 ion channel activity. However, the M2HATM and ΔM2TMCYT viruses did not replicate in mice.

**FIG. 6.** Growth curves of ΔM2TMCYT (□) and wild-type (●) WSN-UdM viruses. MDCK cells were infected with virus at an MOI of 0.01 and incubated at 37°C (A) or 33°C (B). At the indicated times after infection, the virus titer in the supernatant was determined. The values are means of triplicate experiments. The SD is less than 0.40 for each sample.

**FIG. 7.** Incorporation of M2 mutants into influenza virions. Purified viruses were lysed in sample buffer. Viral proteins were treated with 2-mercaptoethanol, separated by SDS–15% PAGE, transferred to a polyvinylidene difluoride membrane, and detected with the 14C2 anti-M2 monoclonal antibody and anti-WSN NP monoclonal antibody. Molecular masses of the marker proteins are shown on the left (in kilodaltons [K]).

**TABLE 2.** Replication of M2 mutants in mice*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean titer (log$_{10}$ PFU/g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nasal turbinate</td>
</tr>
<tr>
<td>Wild type</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>M2V27T</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>M2A30P</td>
<td>NR</td>
</tr>
<tr>
<td>M2S31N</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>M2del29-31</td>
<td>NR</td>
</tr>
<tr>
<td>M2HATM</td>
<td>NR</td>
</tr>
<tr>
<td>ΔM2TMCYT</td>
<td>NR</td>
</tr>
</tbody>
</table>

* Five-week-old female BALB/c mice (n = 4), anesthetized with isoflurane, were infected intranasally with 50 μl of virus (5 × 10³ PFU). Virus titers in organs were determined 3 days after infection with MDCK cells.

b NR, virus not recovered from any of the infected mice (less than 10² PFU/g).

c Mice were infected intranasally with 50 μl of virus (7 × 10² PFU).
Since these mutant viruses grow substantially more slowly than the wild-type virus, they may be rapidly eliminated from the organs by host defense mechanisms, including the immune system. Thus, these results indicate that ion channel activity promotes efficient viral replication.

The M2 ectodomain is thought to be involved in the incorporation of M2 protein into virions (30). Moreover, deletion of 5 or 10 amino acids from the M2 cytoplasmic tail abrogates viral replication (4), possibly through adverse effects on ion channel activity (48) or perhaps by abolishing the protein’s interaction with other viral components, including M1 protein (52). Thus, the greater attenuation in cell culture of ΔM2TMcyt than of M2HATM suggests a requirement for both the TM and cytoplasmic domains of M2, and perhaps the ectodomain (30), to achieve maximally efficient viral replication.

M2 ion channel activity is believed to function at an early stage in the viral life cycle, between the steps of host cell penetration and uncoating of viral RNA. Zhironov (54) reported that low pH induces the dissociation of M1 protein from viral RNPs in vitro. This observation led others to suggest that the introduction of protons into the interior of virions through M2 ion channel activity in the endosomes is responsible for M1 dissociation from RNP (16). If so, how could mutants with defects in ion channel activity replicate at all? Immunoelectron microscopy of the HA protein in virosomes exposed to low pH demonstrated that, in the absence of target membranes, the N-terminal fusion peptide of the HA2 subunit is inserted into the same membrane site where HA is anchored (50). Therefore, the fusion peptide of the HA might be inserted into the viral envelope, forming pores in the viral membrane that permit the flow of protons from the endosome into the virus’s interior, leading to disruption of RNP-M1 interaction and hence to appreciable viral replication.

What is the origin of the M2 ion channel in influenza A virus? M2 ion channel activity was originally discovered in studies of the FPV Rostock strain (43), which has an intracellularly cleavable HA (29, 43, 46). In this strain, the HA undergoes a low-pH-induced conformational change in the trans-Golgi network in the absence of M2 ion channel activity, which raises the pH in this compartment. Hence, in the past, influenza A viruses may have harbored an M2 protein that promoted an increase in the pH of the trans-Golgi network, to a level that prevents conformational changes in the intracellularly cleavable HA. As influenza A viruses without intracellularly cleavable HAs began to appear, there was less selective pressure to maintain high ion channel activity associated with the M2 protein. Although decreased, this ion channel activity may have been sufficient to permit M1 to dissociate from RNP. In fact, ion channel activity differs markedly among the M2 proteins of currently recognized viruses. For example, to display the same ion channel activity as FPV Rostock virus (containing intracellularly cleavable HA), fivefold more M2 protein from human Udorn virus (containing intracellularly uncleavable HA) is needed (46). Conversely, the HAs of some influenza A viruses have changed from intracellularly uncleavable to cleavable during replication in chickens (19, 20, 22), suggesting that M2 protein with limited ion channel activity can acquire greater activity once a switch to intracellularly cleavable HA has occurred.

The M2HATM virus, although replicating reasonably well in cell culture, was highly attenuated in mice, raising the possibility of its use in the production of live vaccines. Cold-adapted live vaccines, now in clinical trials (25), hold considerable promise for use in the general population (38, 39, 40). The major concern is that the limited number of attenuating mutations in such vaccines (6, 17) could permit the generation of revertant viruses. Abolishing M2 ion channel activity, for example, by replacing the M2 TM domain with that from the HA, would greatly reduce the likelihood of the emergence of revertant viruses. Thus, by using the reverse-genetics system described in this report, one could generate influenza viruses with modified viral genes, as a first step in the production of safe live influenza vaccines.

To date, five viral proteins have been reported to act as ion channels: M2 of influenza A virus, NB of influenza B virus, Vpu and Vpr of human immunodeficiency virus type 1 (HIV-1), and Kcv of choleliths virus (12, 31, 32, 33, 37, 43, 44, 45). Since the replication strategies of influenza type A and B viruses are very similar, NB ion channel activity is also thought to play a role at an early stage of the viral life cycle, although this protein still lacks a demonstrated function in viral replication. Although the Vpu gene of HIV-1 can be deleted without completely abrogating HIV-1 replication in vitro (5, 23, 41, 42), the Vpu protein enhances the release of virus particles from cells (36, 41, 47). Vpr, another auxiliary HIV-1 protein, plays an important role in viral replication (8). Choleliths virus PBCV-1 encodes a functional K⁺ channel protein, Kcv, which is important in the virus life cycle (33). On balance, the available data indicate that viral protein ion channel activities are integral parts of the viral life cycle and promote efficient viral replication.

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