Tyrosines in the Influenza A Virus M2 Protein Cytoplasmic Tail Are Critical for Production of Infectious Virus Particles

Michael L. Grantham,1 Shaun M. Stewart,1,2 Erin N. Lalime,1 and Andrew Pekosz1*

W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins University, Bloomberg School of Public Health, 615 North Wolfe Street, Suite 5132, Baltimore, Maryland 21205,1 and Division of Biology and Biomedical Sciences, Washington University in Saint Louis, Campus Box 8226, 660 South Euclid Avenue, Saint Louis, Missouri 631102

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The cytoplasmic tail of the influenza A virus M2 protein is required for the production of infectious virions. In this study, critical residues in the M2 cytoplasmic tail were identified by single-alanine scanning mutagenesis. The tyrosine residue at position 76, which is conserved in >99% of influenza virus strains sequenced to date, was identified as being critical for the formation of infectious virus particles using both reverse genetics and a protein trans-complementation assay. Recombinant viruses encoding M2 with the Y76A mutation demonstrated replication defects in MDCK cells as well as in primary differentiated airway epithelial cell cultures, defects in the formation of filamentous virus particles, and reduced packaging of nucleoprotein into virus particles. These defects could all be overcome by a mutation of serine to tyrosine at position 71 of the M2 cytoplasmic tail, which emerged after blind passage of viruses containing the Y76A mutation. These data confirm and extend our understanding of the significance of the M2 protein for infectious virus particle assembly.

Influenza A virus is a member of the Orthomyxoviridae and contains a segmented, negative-sense RNA genome that codes for 10 or 11 proteins (10). The integral membrane protein M2 is the viral ion channel protein and is required during virus entry (23) as well as for the production of infectious virus particles (8, 11, 12). The M2 protein forms a disulfide-bonded tetramer and contains 97 amino acids, 54 of which are predicted to be in the cytoplasmic tail of the protein (5, 9).

After endocytosis of a virus particle, the M2 protein in the envelope of the virion is believed to shuttle hydrogen ions from the lumen of the endosome into the interior of the virion. This disrupts interactions between viral proteins and allows for the dissociation of the viral ribonucleoprotein complexes (vRNPs) from the site of virus-endosome membrane fusion. The released vRNPs can then associate with the nuclear transport machinery and translocate to the nucleus, where RNA replication occurs (10).

During virus assembly, sequences in the M2 cytoplasmic tail are required for incorporation of nucleoprotein (NP) and vRNPs into progeny virions. When the M2 cytoplasmic tail is truncated by 28 amino acids, progeny virions contain reduced amounts of NP, reduced amounts of some viral RNA (vRNA) segments, and drastically reduced infectivity (12). Using a trans-complementation system consisting of a functionally M2-null virus and cell lines that stably express M2 proteins containing four adjacent alanine substitutions, the region of the M2 cytoplasmic tail important for infectious virus production was narrowed to an 8-amino-acid region (residues 70 to 77) or a 4-amino-acid region (residues 74 to 77), depending on the strain of virus used (11). Consistent with previous experiments using truncated variants of M2, the progeny virions that contained M2 with scanning alanine mutations in these regions also exhibited reduced incorporation of NP and vRNA. Together, these data suggested that one or more of the amino acids from residues 70 to 77 of the M2 cytoplasmic tail were required for incorporation of vRNPs into progeny virions and therefore required for infectivity.

Using single-alanine scanning mutagenesis, an individual amino acid (tyrosine 76) was identified as critical for the production of infectious virus and the incorporation of NP into progeny virions. A revertant virus encoding a tyrosine for serine substitution at position 71 of the M2 cytoplasmic tail was identified which was able to restore the infectivity of recombinant viruses encoding Y76A, reaffirming the importance of the M2 cytoplasmic tail during the production of infectious influenza virus particles.

MATERIALS AND METHODS

Plasmids and mutagenesis. Mutagenesis of the M2 cDNA was achieved through overlap PCR and ligation of the product into the vector pCAGGS (18). Individual codons were changed to create single amino acid substitutions at each position from 70 to 77. The plasmids pHH21-M-Udorn-M2Y76A (where M2Y76A is the M2 protein with the Y76A mutation) and pHH21-M-WSN-M2Y76A were generated by site-directed mutagenesis using the plasmids pH21-M-Udorn and pH21-M-WSN (16, 23), respectively, as a template. Primer sequences are available upon request. The open reading frames (ORFs) of all plasmids were confirmed by sequencing.

Cells. Madin-Darby canine kidney (MDCK) cells and human embryonic kidney cells (293T) were cultured in Dulbecco’s modified Eagle medium (DMEM-Sigma) containing 10% fetal bovine serum (Atlanta Biologicals, Inc.), 100 U of penicillin/ml (Invitrogen), 100 μg of streptomycin/ml (Invitrogen), and 1 mM sodium pyruvate (Sigma) at 37°C and 5% CO2.

All cell lines that stably express M2 or M2 mutants were cultured in medium identical to that used for wild-type (wt) MDCK cells except that it was supplemented with puromycin (7.5 μg/ml; Sigma) and amantadine HCI (5 μM; Sigma). Stable cell lines expressing M2 were generated by cotransflecting MDCK cells

* Corresponding author. Mailing address: W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins University, Bloomberg School of Public Health, 615 North Wolfe Street, Suite 5132, Baltimore, MD 21205, USA. Phone: (410) 502-9306. Fax: (410) 955-0105. E-mail: apekosz@jh.edu.

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with plasmids expressing a puromycin resistance gene (pBABE) (13) and an M2 cDNA expression vector in six-well plates. Two days posttransfection, the cells were trypsinized, placed under puromycin selection (7.5 μg/ml), and cloned either by limiting dilution or by plating cells in a 100-mm dish and picking individual colonies. Cells were screened for M2 expression either by Western blot analysis of whole-cell lysates or by indirect immunofluorescence of live cells for cell surface-expressed M2, followed by detection of positive clones with a fluorescent plate reader.

**Viruses.** Wild-type viruses were propagated on MDCK cells in DMEM containing 4 μg/ml N-acetyltirosin (Sigma), 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 0.3% bovine serum albumin (BSA; Calbiochem or Sigma). The wild-type viruses used in this study were rUdorn (a recombinant virus derived from A/UDorn/72) and rWSN (a recombinant virus derived from A/WSN/33) and have been described previously (16, 23).

Three of the functionally M2-null viruses (rUdorn M2Stop, rWSN M2Stop, and rWSN M1Udorn M2Stop) have been described previously (11). rUdorn M1WSN M2Stop virus contains seven segments from the A/UDorn/72 strain, segment 7 from the A/WSN/33 strain, and stop codons at residues 25 and 26 of the M2 ORF. All of the functionally M2-null viruses were propagated on MDCK cells that express an amantadine-sensitive mutant of WSN M2 (M2WSN-N31S). The recombinant viruses that encode amino acid substitutions in M2 were generated by replacing the plasmid that codes for the wild-type M segment with ones that encode M2 with the desired mutation in the 12-plasmid reverse genetics system (23). Viruses were rescued as described previously (12, 23) with the exception that transfected cells were cocultured with M2WSN-N31S-expressing MDCK cells (MDCK-M2WSN-N31S cells) when the resultant virus was suspected to have a defect in replication. Viruses were plaque purified on MDCK-M2WSN-N31S cells, and the plasmid collection of the M2 segment was sequenced to ensure the presence of the desired mutations and the absence of any spurious mutations.

**Infection of tissue culture cells.** Low-multiplicity growth curves were carried out in triplicate by infecting confluent cell monolayers in 24-well plates at a multiplicity of infection (MOI) of 0.001 50% tissue culture infective doses (TCID50) per cell. Cells were incubated with virus diluted in DMEM containing 4 μg/ml N-acetyltirosin, penicillin, streptomycin, and 0.3% BSA for 1 h at room temperature, the inoculum was aspirated, and the medium was replaced. At the times indicated in the figures, the supernatant was removed and stored at −80°C. The amount of infectious virus in each sample was determined by TCID50 assay as described previously (12) using MDCK cells for samples that contain wild-type viruses and MDCK-M2WSN-N31S-expressing cells for viruses encoding mutated M2 proteins.

High-multiplicity infections were carried out in T75 flasks (MOI of 3) as described previously (3). Cells were incubated with virus diluted in DMEM containing penicillin and streptomycin at room temperature for 1 h. The inoculum was removed, and the cells were washed three times with phosphate-buffered saline (PBS). DMEM containing penicillin and streptomycin was added, and the flasks were incubated at 37°C in 5% CO2 for 15 h. Supernatants were centrifuged at 500 × g for 10 min to remove cell debris. Virus particles were pelleted through a 20% sucrose gradient in a Sorvall TH641 rotor at 118,000 × g for 1 h at 4°C. The pellets were resuspended in PBS, an aliquot was removed for determination of infectious titer, and 4× SDS-PAGE loading buffer was added to the remaining sample for Western blot analysis.

**Western blotting.** Samples were analyzed by Western blot analysis as described previously (3, 12). Polypeptides were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Immobilon-FL; Millipore). All antibodies were diluted in PBS with 5% skim milk powder and 0.3% Tween 20. The antibodies used in this study were 14c2 (anti-M2 monoclonal antibody at 1:1,000) (25) and anti-A/UDorn/72 virus goat serum (1:500) (26). Antibodies were detected using species-specific secondary antibodies conjugated to Alexa Fluor 647 (1:500; Invitrogen), and the blots were imaged using an FLA-5000 phosphorimager (FujiFilm). Bands were quantitated using Multi Gauge software, version 3.0 (GraphPad Software Inc.).

**Fluorescence microscopy.** MDCK cells were infected and stained for influenza A/WSN/33 expression to analyze the amount of hemagglutinin (HA) in the sample.

**Immunofluorescence microscopy.** MDCK cells were infected and stained for immunofluorescence microscopy as described previously (3). MDCK cells were grown on tissue culture-treated glass coverslips and infected on the fifth day after reaching confluence with 5×105 TCID50 per coverslip. At 15 h postinfection (hpi) the cells were fixed and incubated with goat anti-H3 serum raised against HA from A/Aichi/2/68 (1:500; V-314-591-157; National Institute of Allergy and Infectious Diseases), followed by donkey anti-goat IgG conjugated with Alexa Fluor 555 (1:500; Invitrogen). All antibodies were diluted in DMEM with 5% fetal bovine serum. Cells were fixed with 2% paraformaldehyde, and coverslips were mounted using ProLong Gold antifade reagent (Invitrogen).

Samples were imaged with a Nikon Eclipse 90i microscope. In each experiment, images were taken of 10 nonoverlapping, adjacent fields of view (magnification of ×20). Infected cells were then scored for whether they showed filaments on their cell surfaces, and the percentage of infected cells with filaments in each field of view was calculated.

**YFP fluorescence assay.** M2 protein channel activity was tested using a previously published method (15) with modifications. Briefly, the pH-dependent fluorescence of yellow fluorescent protein (YFP) was used as an indicator of cytoplasmic pH (22). Plasmids encoding cDNA constructs for YFP and M2 were cotransfected into 293T cells. The next day, the cells were detached from the plate, resuspended in neutral (pH 7.4) or acidic buffer (pH 5.5), and immediately analyzed by flow cytometry. Fluorescence readings of 10,000 cells were taken every 15 s. The percent change in the mean fluorescence intensity (MFI) of each sample was then plotted versus time. The ion channel activity was blocked with the addition of amantadine (50 μM) to the medium at approximately 6 h posttransfection, and amantadine was included during the analysis by flow cytometry.

**Generation and infection of primary mTEC cultures.** Primary mouse thymus epithelial cell (mTEC) cultures were generated and infected as described previously (3, 17, 21). Cultures were infected with 3,300 TCID50/well, and at the times indicated in the figures, the apical supernatant in each well was harvested and stored at −80°C. The amount of infectious virus in each sample was determined as described above for experiments using MDCK cells. The basolateral medium in each well was not harvested but was replaced with fresh medium every 2 days.

**Statistical analysis.** Low-multiplicity growth curves were compared to wild-type control curves using two-way analysis of variance (ANOVA) with repeated measures. Western blot analyses, infectious virus production from high-multiplicity infections, and the percentage of infected cells with filaments were compared using t tests. All statistical analyses were done using Prism, version 4.0 (GraphPad Software Inc.).

### RESULTS

**Generation of cell lines stably expressing M2 mutants.** Previous work indicated that a region from amino acid 70 to 77 was important for the production of infectious virus of the Udorn strain (A/UDorn/72). A smaller set of amino acids within this region (amino acids 74 to 77) was also important for the production of infectious virus of the WSN strain (A/WSN/33) (11). To identify individual key amino acids within this region a trans-complementation system was used. Single amino acid substitutions were made in the WSN M2 of a cDNA expression vector, and stable cell lines were established for each mutant.

The cell lines were characterized for total M2 expression by Western blot analysis using an anti-M2 monoclonal antibody (Fig. 1A and B). Infectious virus production requires minimal amounts of M2 expression (12), and although expression levels showed some variation, all cell lines expressed more than the threshold amount required. Furthermore, flow cytometric analysis indicated that for each cell line, more than 90% of the cells exhibited M2 expression on the cell surface (data not shown). M2 oligomerization was examined by Western blot analysis carried out under nonreducing conditions (Fig. 1C and D). Each of the scanning alanine mutants was able to form disulfide-linked dimers and tetramers to an extent that was similar to wild-type M2.

**Complementation of viruses that do not express M2.** Amino acids 70 to 73 in the M2 cytoplasmic tail were critical for supporting infectious virus production of rUdorn M2Stop but not rWSN M2Stop (11). Cell lines expressing Udorn M2 proteins in which amino acids 70 to 73 were mutated individually to alanine were used for low-MOI growth curves with rUdorn M2Stop or rWSN M2Stop (MOI of 0.001). Supernatants from cell lines expressing wild-type M2 and infected with rUdorn M2Stop contained more than 1×107 TCID50/ml of infectious
virus by 2 days postinfection (dpi), while cells that did not express M2 produced little (if any) detectable virus (Fig. 2A). Mutation of amino acid 70 had no adverse effect on the amount of infectious virus produced, while mutation of amino acid 71, 72, or 73 reduced infectious virus production (Fig. 2A). The mutation that had the greatest effect was the M72A substitution. At 2 dpi, cells expressing this mutant produced greater than 1,000-fold less infectious virus than cells that expressed wild-type M2. When these cell lines were infected with rWSN M2Stop, none of mutations between amino acids 70 and 73 had a statistically significant effect on the amount of infectious virus produced. This result is consistent with previous experiments examining strain-specific effects on infectious virus production of mutations in this region of the M2 protein (12).

M2 amino acids 74 to 77 were shown to be required for infectious virus production in a strain-independent manner (12). Alanine substitutions at positions 75, 76, and 77 resulted in significant decreases in infectious virus production compared to wild-type M2 after infection with rUdorn M2Stop (Fig. 2C). The reduction was greatest with a Y76A substitution, leading to an almost complete loss in infectious virus production of mutations in this region of the M2 protein (12).

Similar results were observed after infection with rWSN M2Stop (Fig. 2D), but the magnitude of the reduction in infectious virus production was not as great as that seen after infection with rUdorn M2Stop. Since the M2Y76A mutation resulted in the most severe phenotype, subsequent work concentrated on this mutation.

To determine whether the genetic background of the mutated M2 protein affects the complementation of M2Stop viruses, a cell line was generated that expresses the M2 protein from A/WSN/33 in which tyrosine 76 was replaced with alanine. In this case, the protein also contains a mutation which confers amantadine sensitivity (substitution of asparagine to serine at position 31) (4, 23) so that amantadine could be added during routine cell culture to reduce the potentially toxic effects of constitutive M2 expression. The surface expression and oligomerization of M2WSN-N31S/Y76A was confirmed and was similar to that of M2WSN-N31S (data not shown). As shown in Fig. 2E and F, the phenotype of the M2WSN-N31S/Y76A mutant was consistent with that seen with M2Udorn-Y76A, indicating that the Y76A mutation had a similar effect in either the Udorn- or WSN-derived M2 proteins. When infected with rUdorn M2Stop, the cells produced little infectious virus at any time point tested. When this cell line was infected with rWSN M2Stop, more infectious virus was produced than when cells were infected with rUdorn M2Stop; however, the supernatants still contained less infectious virus than supernatants from infected M2WSN-N31S-expressing cells.

Previous experiments suggest that at least some of the observed differences in the M2 cytoplasmic tail sequence requirements between the Udorn and WSN strains of virus can be mapped to the M1 protein (1, 11). MDCK-M2Udorn-Y76A cells were infected either with a rUdorn M2Stop virus that encodes the M1 protein from the WSN strain (rUdorn M1WSN M2Stop) (Fig. 3A) or with an rWSN M2Stop virus that encodes the M1 protein from the Udorn strain (rWSN M1Udorn M2Stop) (Fig. 3B). Replacing the Udorn M1 protein with the WSN M1 protein led to increased virus replica-
While replacing the WSN M1 protein with the Udorn M1 protein reduced infectious virus production (compare Fig. 3B and 2F), these data indicate that the extent of the defect in infectious virus production for the M2Y76A mutant is dependent upon sequences present within the M1 protein.

**Ion channel activity of M2Y76A.** Previous data indicated that many mutations in the cytoplasmic tail of the M2 protein (e.g., truncation of M2) had no deleterious effect on ion channel activity (24). In order to determine if the M2Y76A protein displayed altered ion channel activity, changes in YFP fluorescence induced by cytosolic pH fluxes were assessed.
As shown in Fig. 4A, exposure of cells expressing the M2 protein to low pH resulted in a decrease in the MFI over time, reaching a maximum within 7.5 min. This decreased MFI was not seen in the absence of M2 expression. The decrease in MFI was not as great in M2-expressing cells treated with amantadine, indicating that the effect was specific to the M2 ion channel activity. When the ion channel activity of M2Y76A was tested (Fig. 4B), the change in MFI was pH dependent, and the magnitude of the change in MFI was indistinguishable from that of wt M2 Udorn, indicating that there is no discernible defect in the ion channel activity of M2Y76A compared to wt M2, and therefore the loss of infectious virus production due to this mutation was not the result of defective ion channel activity.

**Generation of M2Y76A viruses.** To verify the effect of M2Y76A that was observed in the trans-complementation system, the mutation was introduced into the genomes of both the Udorn and WSN strains by reverse genetics, and the resulting viruses were propagated on cells that express wild-type M2. MDCK cells were infected at a low multiplicity, and the amount of infectious virus in infected cell supernatants was determined (Fig. 5A and B). Consistent with the results from complementation assays, viruses carrying the M2Y76A mutation had a statistically significant reduction of infectious virus production in MDCK cells. The defect seen in rUdorn-M2Y76A was particularly severe since infectious virus was not detected at any of the time points tested. The rWSN-M2Y76A virus replicated better than rUdorn-M2Y76A, and the extent of attenuation was on the order of that seen when M2WSN-N31S/Y76A-expressing cells were used to complement the replication of rWSN M2Stop (Fig. 2F).

To verify that the defect in infectious virus production was due to the introduction of the M2Y76A mutation and not to the presence of an unidentified spontaneous mutation elsewhere in the virus genome, growth of the recombinant viruses was examined on cells that express M2WSN-N31S (Fig. 5C and D). For both virus strains, the M2Y76A mutant replicated as well as the wild-type control on the M2-complementing cell line, indicating that the defect in infectious virus production for both of these mutants was due solely to the presence of the M2Y76A mutation in the protein and was not a result of changes in the genomic RNA that may have disrupted RNA packaging signals.

**Tyrosine 76 is required for incorporation of NP into virus particles.** To determine whether the Y76A mutation affected the incorporation of proteins into progeny virus particles, MDCK cells were infected (MOI of 3) in the absence of tryp-
sin, and the supernatant was collected at 15 hpi. Virus particles were concentrated by ultracentrifugation, and Western blot analysis was used to examine the viral structural proteins.

The amount of HA did not vary greatly between the rUdorn and rUdorn-M2Y76A viruses, suggesting that there was no defect in the total amount of virus particles produced (Fig. 6A). The amount of infectious virus was reduced by more than 100-fold, indicating that the rUdorn-M2Y76A virus was capable of producing virus particles, but these particles were less infectious than those of rUdorn.

The amounts of NP, M1, and M2 shown in Fig. 6A were quantified after normalization to total HA (Table 1). Although there was a tendency for less M1 to be incorporated into rUdorn-M2Y76A particles, this difference was not always statistically significant between experiments. Both NP and M2 were seen as doublet bands in Western blot analyses. This may be due to the cleavage of these two proteins by cellular caspases, as described previously (27). The slower-migrating form of NP (representing full-length NP, which we designated NPa) was present at 2.6-fold-lower levels (P < 0.05) in the rUdorn-M2Y76A virus particles than in rUdorn virus. Furthermore, the total amount of NP incorporated into rUdorn-M2Y76A virus particles was less than half the amount incorporated into wild-type virus particles. The total amount of M2 incorporated was similar, indicating that the change in virus infectivity was not a result of reduced incorporation of the M2Y76A protein into virions. Viral proteins, in particular NPa, were present at equivalent amounts in virus-infected cell lysates (Fig. 6A), suggesting that the packaging, not the absence of NP, was the limiting factor in rUdorn-M2Y76A-infected cells.

To determine if expression of M2Y76A in trans could alter NP protein packaging, MDCK cells expressing either wild-type M2 or M2Udorn-Y76A were infected with rUdorn M2Stop in the absence of trypsin. At 15 hpi, the supernatants were harvested and analyzed for viral protein incorporation and infectious virus titer. However, virus particles containing M2Y76A still packaged nearly 2-fold less NPa than virus particles complemented with wild-type M2 (Fig. 6C and Table 1). Consistent with the reduced incorporation of NPa, the amount of infectious virus produced by the cells expressing M2Y76A was 10-fold less than that produced by cells expressing wild-type M2 (Fig. 6D). Interestingly, rUdorn M2Stop virus particles harvested from MDCK cells also displayed reduced incorporation of NPa, indicating that the absence of M2 also led to altered incorporation of NPa. In contrast to the data with recombinant viruses, the levels of M1 protein incorporation into rUdorn M2Stop viruses grown on M2Y76A-expressing cells was consistently less than that observed in virions purified from cells expressing wild-type M2 (Table 1). These data suggest that incorporation of large amounts of M2Y76A is not sufficient to overcome the defect in the production of infectious rUdorn-M2Y76A particles.

To determine whether wild-type M2 could complement the...
defect in NP α incorporation of rUdorn-M2Y76A, virus was grown on MDCK cells expressing M2Udorn (MDCK-M2Udorn), and Western blot analysis was carried out as described above (Fig. 6E). After normalization to the HA0 protein, there were no significant differences in the amounts of viral proteins packaged into virions for either virus strain (Table 1). Furthermore, there was a slight but significant increase in the amount of infectious rUdorn-M2Y76A produced com-

FIG. 6. Mutations in the M2 protein cytoplasmic tail alter virion polypeptide composition. The indicated viruses were used to infect MDCK cells at a high MOI. At 15 hpi, cell lysates were harvested for Western blotting (A), and virus particles purified from infected cell supernatants were analyzed for polypeptide composition by Western blotting (A) and for infectious virus by TCID₅₀ assay (B). The indicated cells were infected with rUdorn M2Stop virus at a high MOI for 15 h, and virus particles were purified from infected cell supernatants and analyzed for polypeptide composition by Western blotting (C) and for infectious virus by TCID₅₀ assay (D). The indicated viruses were used to infect MDCK-M2Udorn cells at a high MOI. At 15 hpi, virus particles were purified from infected cell supernatants and analyzed for polypeptide composition by Western blotting (E) and for infectious virus by TCID₅₀ assay (F). **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
The supernatant contained a low level of infectious virus (4.6 x 10^3 TCID$_{50}$/ml; referred to as rUdorn-M2Y76A; Table 1) which was able to form plaques on MDCK cells, unlike rUdorn-M2Y76A. A segment coding region of five plaque-purified viruses was sequenced, and in addition to the Y76A mutation, a serine-to-tyrosine mutation was present at position 71 of the M2 ORF of each virus. Two plaque-purified viruses were expanded and used in low-MOI growth curve experiments on MDCK cells (Fig. 7A). Both viruses had growth kinetics and reached peak titers that were nearly identical to those of the wild-type virus.

To determine whether the M2S71Y mutation was sufficient to restore replication of rUdorn-M2Y76A, recombinant viruses containing the suppressor mutation were generated and characterized. Virus containing either the M2S71Y mutation alone or both S71Y and Y76A mutations (M2S71Y/Y76A) were generated and analyzed by a low-MOI growth curve (Fig. 7B). M2S71Y-encoding viruses replicated to significantly greater titers than the rUdorn M2Y76A virus, indicating that the S71Y mutation alone could restore the replication of that virus on MDCK cells. rUdorn-M2S71Y replicated slightly faster than wild-type virus and produced about 1 log more infectious virus at 1 dpi. rUdorn-M2S71Y/Y76A replicated slightly more slowly than rUdorn and produced about 1 log less infectious virus at 1 dpi.

The structural proteins packaged into virions were examined following infection of MDCK cells at a high MOI, and viruses encoding S71Y were indistinguishable from wild-type virus, indicating that the packaging of NPa was restored in recombinant viruses containing the suppressor mutation. Together, these data indicate that tyrosine 76 is required for incorporation of NP into virions and to maintain infectious virus production but also to restore the incorporation of wild-type M2 is able not only to complement the defect caused by the Y76A mutation. The S71Y mutation alone could restore the replication of that virus on MDCK cells. rUdorn-M2S71Y replicated slightly faster than wild-type virus and produced about 1 log more infectious virus at 1 dpi. rUdorn-M2S71Y/Y76A replicated slightly more slowly than rUdorn and produced about 1 log less infectious virus at 1 dpi.

**Generation and characterization of an M2Y76A suppressor mutant.** To determine if suppressor mutations that could restore infectivity to rUdorn-M2Y76A viruses could be identified, MDCK cells were infected with rUdorn-M2Y76A. At 60 hpi with an MOI of 0.1, approximately 50% of the cells exhibited cytopathic effect (CPE). The supernatant contained a low level of infectious virus (4.6 x 10^3 TCID$_{50}$/ml; referred to as rUdorn-M2Y76A) which was used to infect fresh MDCK cells (MOI of 0.001). At 48 hpi, 100% of the cells exhibited CPE, and infectious virus titers in the supernatant were substantially increased (rUdorn-M2Y76AP2; 3.2 x 10^6 TCID$_{50}$/ml). The resulting virus was able to form plaques on MDCK cells, unlike rUdorn-M2Y76A. A segment coding region of five plaque-purified viruses was sequenced, and in addition to the Y76A mutation, a serine-to-tyrosine mutation was present at position 71 of the M2 ORF of each virus. Two plaque-purified viruses were expanded and used in low-MOI growth curve experiments on MDCK cells (Fig. 7A). Both viruses had growth kinetics and reached peak titers that were nearly identical to those of the wild-type virus.

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<th>Figure</th>
<th>Protein</th>
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<tr>
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$^a$ Virus proteins were quantified, and amounts are expressed relative to the amount of HA protein. Three to six independent experiments were averaged, and the standard error is shown. NA, not applicable; M2a, full-length M2; $^*$, P ≤ 0.05; **, P ≤ 0.01.
in tissue culture (14, 19, 20). To determine whether the M2Y76A and M2S71Y mutations affected the formation of filamentous influenza, MDCK cells were infected and analyzed by immunofluorescence microscopy. Qualitatively, cells infected with rUdorn-M2Y76A produced filaments that appeared shorter and less abundant on a per-cell basis than those produced by rUdorn (Fig. 8A). However, rUdorn-M2S71Y and rUdorn-M2S71Y/Y76A both produced filaments that were very similar to those seen in rUdorn-infected cells. As shown in Fig. 8B, rUdorn-M2Y76A infections produced detectable filaments on the surface of a smaller proportion of infected cells than rUdorn, rUdorn-M2S71Y, or rUdorn-S71Y/Y76A. These data indicate that, along with a defect in NPa incorporation into progeny virions, rUdorn-M2Y76A exhibits a defect in filament formation on the surface of infected cells and that this defect can be overcome by the M2S71Y mutation.

**Replication in primary, differentiated mTEC cultures.** To determine if the Y76A and S71Y mutations could alter influenza virus replication in respiratory epithelial cells, differentiated mouse trachea epithelial cell (mTEC) cultures were prepared as described previously (3, 17, 21) and infected with rUdorn, rUdorn-M2Y76A, rUdorn-M2S71Y, rUdorn-M2S71Y/Y76A, rWSN, and rWSN-M2Y76A. As shown in Fig. 9, the phenotypes seen previously in MDCK cells were reproduced in the primary mTEC cultures but to a much greater extent. Cultures infected with rUdorn-M2S71Y produced greater than 100-fold more infectious virus on day 2 postinfection than cultures infected with rUdorn, and peak virus titers occurred considerably earlier. Cultures infected with rUdorn-M2S71Y/Y76A reached peak titer at a similar time as cultures infected with rUdorn, but contained about 100-fold less infectious virus. However, supernatants from rUdorn-M2S71Y/Y76A-infected cells still contained at least 100-fold more infectious virus than rUdorn-M2Y76A-infected cultures. Similarly, the replication defect of rWSN-M2Y76A was greater in mTEC cultures (Fig. 9B) than in MDCK cells (Fig. 5B). Together, these data indicate that the phenotypes seen in MDCK cells hold true but are significantly more pronounced in primary, differentiated airway epithelial cell cultures. These data confirm a central role for M2Y76A in infectious virus production.

**FIG. 7.** Identification of a suppressor mutation for M2Y76A. (A) Supernatants from rUdorn-M2Y76A-infected MDCK cells were passaged twice on MDCK cells (rUdorn-M2Y76AP2). Viruses from the resulting infected cell supernatants were plaque cloned on MDCK cells (pq2 and pq4) and then used to infect MDCK cells at a low MOI. Infectious virus titers in infected cell supernatants were determined at the indicated times. (B) Recombinant influenza viruses bearing the indicated mutations were used to infect MDCK cells at a low MOI. Infectious virus titers in the infected cell supernatants were determined at the indicated times. The dotted lines indicate the limits of detection, and asterisks indicate statistically significant differences compared to wild-type control curves. MDCK cells were infected at a high MOI, and virus particles were purified from infected cell supernatants at 15 hpi and analyzed by Western blotting (C) or for infectious virus titer (D). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
and the standard error is shown. M2a, full-length M2; amount of HA protein. Three or four independent experiments were averaged for replication has also been identified in this region. Synonymous of the M2 protein, an RNA sequence that is important truncated after residue 75 (8). In addition to important function, disruption of the sequence between residues 70 to 77 led to a defect in the specific incorporation of NP into progeny virions (11, 12, 23). The data described here indicate that amino acid 76 of the M2 cytoplasmic tail is a key residue that is required for incorporation of NP into virus particles and for maintaining infectious virus production. Tyrosine 76 is highly conserved among influenza A isolates, showing conservation in 8,702 of 8,706 sequences examined (data not shown), but it is not yet known how the abundance of NP affects virion infectivity. Incorporation of a full comple-

**TABLE 2. Quantitation of viral proteins incorporated into influenza A virus particles expressing M2 proteins with an S71Y mutation**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Protein</th>
<th>Virus</th>
<th>Amount of protein in MDCK cellsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>7C</td>
<td>Total NP</td>
<td>rUdorn</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2Y76A</td>
<td>0.10 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y</td>
<td>0.16 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y/Y76A</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>NPa</td>
<td>rUdorn</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2Y76A</td>
<td>0.044 ± 0.008*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y/Y76A</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>rUdorn</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2Y76A</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y/Y76A</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Total M2</td>
<td>rUdorn</td>
<td>0.030 ± 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2Y76A</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y</td>
<td>0.021 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y/Y76A</td>
<td>0.017 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>M2a</td>
<td>rUdorn</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2Y76A</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y</td>
<td>0.017 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rUdorn-M2S71Y/Y76A</td>
<td>0.008 ± 0.002</td>
</tr>
</tbody>
</table>

a Virus proteins were quantified, and amounts are expressed relative to the amount of HA protein. Three or four independent experiments were averaged and the standard error is shown. M2a, full-length M2; *, P ≤ 0.05.

**DISCUSSION**

The influenza A virus M2 protein plays a number of key roles in the virus life cycle. The ion channel activity of the protein is required during virus entry to allow proper release of vRNPs from virions, and sequences in the cytoplasmic tail of M2 are essential for the incorporation of NP and vRNPs into progeny virions (11, 12, 23). The data described here indicate that amino acid 76 of the M2 cytoplasmic tail is a key residue that is required for incorporation of NP into virus particles and for maintaining infectious virus production. Tyrosine 76 is highly conserved among influenza A isolates, showing conservation in 8,702 of 8,706 sequences examined (data not shown), but it is not yet known how the abundance of NP affects virion infectivity. Incorporation of a full comple-

![FIG. 8. Mutations at positions 71 and 76 of the M2 cytoplasmic tail alter the production of filamentous virus particles. (A) MDCK cells were infected at a high MOI with the indicated recombinant viruses and analyzed for filamentous particle formation by immunofluorescence microscopy at 15 hpi. The images shown represent a deconvolved series of images acquired with a 40× objective. (B) The number of infected cells and infected cells expressing filamentous virus projections were quantified per field imaged (magnification, ×20) and are expressed as follows: number of infected cells with filaments/number of infected cells × 100. *, P ≤ 0.05; ***, P ≤ 0.001.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/ on July 3, 2017 by guest)
virus particles, the differences in M1 incorporation were not always statistically significant. This does not rule out the possibility that the M2Y76A mutation alters an M1-M2 interaction. In fact, the reduced specific incorporation of M1 into rUdorn M2Stop grown on cells expressing M2Y76A could be a result of overexpression of a variant of M2 with altered M1-M2 interactions. Furthermore, the finding that the extent of the defect in infectious virus production depends on the source of the M1 protein in the virus could be interpreted as further genetic evidence for functionally important M1-M2 interactions. Although the data in this study and the one from Chen et al. appear to be contradictory in terms of the relative importance of M1 and NP (specifically, NPa) incorporation into virions, the differences are most likely due to the methods used to quantitate Western blot analyses. Chen et al. normalized the amount of protein in virions to the amount of M1 in infected cell lysates, and this measures the effects of a given mutation on the general process of virus budding. In this study, similar amounts of HA in samples of rUdorn and rUdorn-M2Y76A suggest that there was no major defect in virus budding. Therefore, the amount of protein in virions was normalized to that of HA in the supernatants. This method accounts for minor differences in general virus budding and quantifies the specific incorporation of each viral protein.

A selection for mutations that suppress the phenotype of rUdorn-M2Y76A yielded a virus that contained an M2S71Y substitution after only two blind passages of the parental virus in MDCK cells. Recombinant viruses containing this mutation and the M2Y76A mutation exhibited nearly wild-type levels of replication in MDCK cells and at least a 100-fold increase over the rUdorn-M2Y76A peak titer on mTEC cultures. Virus containing both M2 mutations contained levels of structural proteins that were indistinguishable from those of wild-type virus, while virus containing only the M2S71Y mutation replicated slightly better on MDCK cells and considerably better on mTEC cultures than wild-type virus.

In addition, the M2S71Y mutation led to a statistically significant increase in the number of infected cells that exhibited HA-positive, cell surface filaments. The significance of this finding is not completely clear since it has been difficult to correlate the mechanism of filament formation with that of the assembly of infectious virus particles. However, increased filament formation may be a sign of more efficient virus assembly in the presence of the M2S71Y mutation. Interestingly, an identical mutation was identified in a screen for viruses that were resistant to the growth-inhibitory effects of the anti-M2 monoclonal antibody 14c2. The resulting virus was also able to form filaments in the presence of 14c2, unlike the parental A/Udorn/72 (20). The mechanism by which that might occur is a matter of investigation but is likely to lead to new insight into the mechanisms required for the assembly of infectious influenza A virus particles.

ACKNOWLEDGMENTS

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FIG. 9. Mutations at positions 71 and 76 of the M2 cytoplasmic tail alter virus replication in mTEC cultures. The indicated recombinant viruses in the rUdorn (A) or rWSN (B) genetic background were used to infect mTEC cultures. Infectious virus titers in the apical washings were determined at the indicated times postinfection. The dotted lines indicate the limits of detection, and asterisks indicate statistically significant differences compared to wild-type control curves. **, $P \leq 0.01$; ***, $P \leq 0.001$. 

*This study was supported by Public Health Service grants AI007417 (M.L.G.), AI061253 (A.P.), and AI053629 (A.P.) from the National Institutes of Allergy and Infectious Diseases. A.P. also acknowledges support from the Eliasberg Foundation and the Marjorie Gilbert Foundation.*
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