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# The Cytoplasmic Tail of Influenza A Virus Neuraminidase (NA) Affects NA Incorporation into Virions, Virion Morphology, and Virulence in Mice but Is Not Essential for Virus Replication

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**In this study, we investigated the role of the conserved neuraminidase (NA) cytoplasmic tail residues in influenza virus replication. Mutants of influenza A virus (A/WSN/33 [H1N1]) with deletions of the NA cytoplasmic tail region were generated by reverse genetics. The resulting viruses, designated NOTAIL, contain only the initiating methionine of the conserved six amino-terminal residues. The mutant viruses grew much less readily and produced smaller plaques than did the wild-type virus. Despite similar levels of NA cell surface expression by the NOTAIL mutants and wild-type virus, incorporation of mutant NA molecules into virions was decreased by 86%. This reduction resulted in less NA activity per virion, leading to the formation of large aggregates of progeny mutant virions on the surface of infected cells. A NOTAIL virus containing an additional mutation (Ser-12 to Pro) in the transmembrane domain incorporated three times more NA molecules into virions than did the NOTAIL parent but approximately half of the amount incorporated by the wild-type virus. However, aggregation of the progeny virions still occurred at the cell surface. All NOTAIL viruses were attenuated in mice. We conclude that the cytoplasmic tail of NA is not absolutely essential for virus replication but exerts important effects on the incorporation of NA into virions and thus on the aggregation and virulence of progeny virus. In addition, the relative abundance of long filamentous particles formed by the NOTAIL mutants, compared with the largely spherical wild-type particles, indicates a role for the NA cytoplasmic tail in virion morphogenesis.**

The neuraminidase (NA) molecule of influenza A virus is a type II membrane glycoprotein which has an uncleaved amino-terminal signal/anchor domain and a six-amino-acid tail (1a). Although the precise location of each of the amino-terminal six polar residues is unknown, they are presumed to be exposed to the cytoplasm (1a, 4). The NA facilitates the mobility of virions by removing sialic acid residues from virus and infected cells during both entry and release from the cells (1a, 15, 21). The ectodomain of NA consists of a stalk and a globular head (1a), with the latter containing the enzyme-active center and major antigenic sites. In contrast to the remainder of the NA, the six-amino-acid cytoplasmic tail is highly conserved among all NA subtypes of influenza A virus. Such extreme conservation suggests a critical role for this domain in the life cycle of the virus.

With the availability of methods to produce transfectant viruses containing genes derived from cloned DNA (reverse genetics [5, 6, 22]), studies have indicated a role for the NA cytoplasmic tail in virus replication (2). Previous attempts to generate a virus containing a deleted NA cytoplasmic tail were not successful (2), suggesting that the mutation was lethal, or perhaps that it affected virus replication to the extent of preventing rescue. However, other transfectant viruses with the NA tail of type B, instead of type A, virus or a point mutation in the NA tail were generated. Mutations in the cytoplasmic tail reduced NA incorporation into virions, a finding that correlated with reduced virulence in mice. Others have shown that NAs with a proline replacement of the initiating methionine, or an addition of proline at the amino terminus of the methionine, continue to function in virus replication (23) but are associated with reduced viral growth (1 to 2 $\log_{10}$). Thus, the cytoplasmic tail domain appears to play an important role in virus replication.

Using reverse genetics, we successfully generated viruses containing a deletion of, and a mutation in, the NA cytoplasmic tail. Here we report the properties of these mutants, emphasizing NA surface expression, NA enzymatic activity, aggregation effects of progeny virions on infected cells, virulence in mice, and virion morphology.

## MATERIALS AND METHODS

**Viruses and cells.** Influenza virus A/WSN/33 (H1N1) (WSN) was obtained from Thomas Chambers (University of Kentucky, Lexington). Masahiro Ueda (The Institute of Public Health, Tokyo, Japan) provided a helper virus (WSN-HK [H1N2]) that was used to rescue the WSN NA gene; it contained the NA gene from influenza virus A/Hong Kong/1/68 (H3N2) and all other genes from WSN (25). Madin-Darby bovine kidney (MDBK) cells were maintained in Eagle's minimal essential medium in the presence of 10% fetal calf serum. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium in the presence of 5% newborn calf serum (NCS).

**Reverse genetics.** Construction of plasmid pT3WSN(NA15), which contains the WSN NA gene flanked by an upstream T3 RNA polymerase promoter sequence and a downstream *Ksp*632I site, was described previously (3), as was construction of a plasmid containing the NOTAIL NA gene (2). Plasmid pT3WSN(CYTPro-Thr) was constructed by oligonucleotide-directed mutagenesis (13), in which Thr was substituted for the NA Pro-3. NA ribonucleoprotein complex was prepared by transcription of pT3WSN(NA15) or its derivatives with T3 RNA polymerase in the presence of nucleoprotein and polymerase proteins (22) after these plasmids had been digested with *Ksp*632I and filled in with Klenow fragment, as described elsewhere (2, 5, 6). The NA ribonucleoprotein

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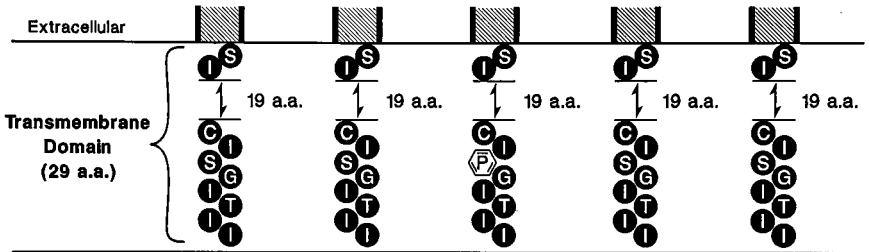


| Virus: | wild-type | NOTAIL/ 4AE | NOTAIL/ 4AF,7CC, 7CD | CYTPro-Ala | CYTPro-Thr |
|---|---|---|---|---|---|
| Rescued: | yes | yes | yes | NO | yes |
| PFU | 8.0 ± 0.15 | 5.6 ± 0.06 | 6.7 ± 0.08 | N.D. | 6.7 ± 0.15 |
| Plaque size (mm diameter): | 2.0 ± 0.5 | 0.5 ± 0.1 | 1.1 ± 0.1 | N.D. | 1.3 ± 0.3 |
| $MLD_{50}$ ($log_{10}$ PFU/ml): | 3.6 | 6.1 | 6.6 | N.D. | N.D. |

FIG. 1. Amino acid (a.a.) sequences and infectivity of wild-type (WSN) and mutant NAs. CYTPro-Ala was previously called NO PRO (2). All NA sequences (ectodomain sequences are not shown) in the mutant viruses were identical to these of the wild-type NA except where indicated. Transfectant viruses were plaque purified three times in MDBK cells and titrated in MDCK cells. N.D., not determined (i.e., transfectant virus was not rescued). Plaque sizes were measured and averaged at 3 days postinfection of MDCK cells. Six-week-old female BALB/c mice were infected intranasally with 50 μl of virus at different dilutions (three mice per dilution), and minimal lethal doses in 50% of the mice ($MLD_{50}$) were calculated 21 days postinfection. The values are means ± standard deviations of three independent experiments.

complex was then transfected into 70 to 90% confluent MDBK cells infected 1 h before transfection with WSN-HK at a multiplicity of infection of 1. Eighteen hours after transfection, transfectant viruses in the supernatant were assayed for plaque formation on MDBK cells (5) and then plaque purified three times in MDBK cells. Viral RNA was extracted from plaque-purified transfectants, used for cDNA synthesis, and sequenced to confirm the identity of each transfectant.

**Purification of transfectant viruses.** Transfectant viruses were grown in MDCK cells containing 0.5 μg of trypsin per ml. Viruses were purified on sucrose gradients (25 and 70% sucrose in phosphate-buffered saline [PBS; pH 7.4] underlay, then 25 to 70% sucrose in PBS continuous gradient) by ultracentrifugation (130,000 × *g* for 1 h at 4°C). Virus was resuspended in PBS and stored in aliquots at −70°C until used.

**NA specific activity.** Viral NA activity was determined by the method of Warner and O'Brien (26) in 0.1 M potassium phosphate buffer (pH 5.9) with 1 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma Chemical Co.) as the substrate. Duplicate reaction mixtures (purified virus and substrate in a total volume of 10 μl) were incubated in U-bottom microtiter plates at 37°C for the indicated times; then the reactions were stopped by the addition of 200 μl of 0.1 M glycine buffer (pH 10.7) containing 25% ethanol. The fluorescence of released 4-methylumbelliferone (4-MU; Sigma Chemical Co.) was determined on a Labsystems Fluoroskan spectrophotometer ($\lambda_{ex}$ = 355 nm; $\lambda_{cm}$ = 460 nm). Viral protein concentration was determined with a Bio-Rad protein assay kit. Specific activities were calculated and expressed as micromoles of 4-MU per microgram of total viral protein.

**Glycoprotein incorporation into virions.** MDBK cells were infected with wild-type or mutant virus. Four hours later, cells were starved of glucose for 30 min and labeled with 0.2 mCi of [$^3$H]mannose (Amersham) for 14 h. Virus in the culture supernatant was purified by centrifugation (1 h) at 130,000 × *g* through 30% sucrose. The virus pellet was disrupted with lysis buffer (50 mM Tris-HCl [pH 7.5], 600 mM KCl, 0.5% Triton X-100). Viral proteins were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) (14).

**Fluorescence-activated cell sorting (FACS).** A confluent monolayer of cultured MDCK cells was infected with virus (multiplicity of infection of 3) for 1 h (37°C), after which cells were washed three times with PBS and incubated at 37°C. At the indicated times postinfection, cells were placed at 4°C, washed twice, and then suspended by limited trypsinization at 37°C. Suspended cells were centrifuged (15,000 × *g*) for 30 s, washed twice, and resuspended in 5% NCS–PBS. Each sample was divided into two aliquots; one was labeled with a 1:50 dilution (in PBS) of anti-WSN NA monoclonal antibody (529/1), and the other was labeled with a pool of anti-WSN HA monoclonal antibodies (162/3, 189/2, 410/2, 523/6, and 524/2). After 30 min at 4°C, cells were washed three times with 5% NCS–PBS and labeled (4°C, 30 min) with a 1:20 dilution of anti-mouse immunoglobulin G-conjugated fluorescein isothiocyanate-labeled antibody (Boehringer Mannheim Biomedicals). The cells were washed twice with 5% NCS–PBS and then fixed in 3.7% paraformaldehyde–PBS at room temperature. Cell sorting was performed with a FACScan (Becton Dickinson) instrument.

**Experimental infection.** Six-week-old female BALB/c mice, anesthetized with methoxyflurane, were infected intranasally with 50 μl of virus at different dilutions (three mice per dilution). The minimal lethal dose in 50% of the mice was determined at 21 days postinfection for each virus.

**Electron microscopy.** Infected cells were washed three times with PBS, fixed with 2% glutaraldehyde in PBS for 2.5 h at 4°C, and postfixed with 2% osmium tetroxide. The fixed cells were dehydrated with increasing concentrations of ethanol from 50 to 100% and embedded in a mixture of epoxy resin. Polymerization was done at 70°C for 48 h. After staining with uranyl acetate and lead citrate, the ultrathin sections were viewed and photographed with a JEOL transmission electron microscope. For cells treated with bacterial neuraminidase, 5.5 mU of purified *Vibrio cholerae* neuraminidase (protease free; GIBCO) was added for 1 h (37°C) before the cells were fixed.

Purified virus was negatively stained as described by Murti et al. (19). Briefly, virions in STE (0.1 M NaCl, 0.05 M Tris, 0.01 M EDTA [pH 7.4]) buffer were adsorbed to carbon-coated electron microscope grids. The grids were rinsed with PBS (pH 7.4), negatively stained with 2% phosphotungstic acid (pH 6.4), and examined with a Philips 301 electron microscope.

## RESULTS

**NOTAIL virus.** Although we were unable to rescue the NOTAIL virus in previous attempts (2), further efforts yielded mutants containing a deletion of the NA cytoplasmic tail. In this study, plaque assays of the transfectant virus were extended to 5 days postinfection (instead of 3 days as in the previous study), at which time only four pinpoint plaques were observed, indicating that the deletion significantly affected virus replication. Sequence analysis revealed that one virus (NOTAIL/4AE) lacked additional mutations in the NA molecule (Fig. 1), while each of the other three viruses (NOTAIL/4AF, -7CC, and -7CD) had a serine 12-to-proline mutation in the transmembrane domain. There were no appreciable differ-

ences in viral growth characterizing these three mutants, and thus NOTAIL/7CC was arbitrarily chosen for further analysis. Although deletion of the NA cytoplasmic tail, with or without the Ser-12-to-Pro mutation, failed to inhibit virus replication entirely, it caused significant reductions in virus growth and plaque diameters (Fig. 1), with NOTAIL/4AE and NOTAIL/7CC growing at levels 2.4 $\log_{10}$ and 1.3 $\log_{10}$ lower than that of wild-type virus. Since the Ser-12-to-Pro mutation (NOTAIL/7CC) increased the replicative ability of viruses (Fig. 1) compared with the NOTAIL mutant without this mutation (NOTAIL/4AE), we conclude that the selective mutation partially compensates for the deletion of the NA cytoplasmic tail.

**Mutations in the NA cytoplasmic Pro residue.** The proline in the third position is highly conserved in both type A and B influenza virus NAs. Previous substitution of this residue with alanine did not result in the generation of infectious virus (2), suggesting a role for proline in virus replication. Nonetheless, with rescue of a NOTAIL virus, we reexamined the impact of a Pro-to-Ala substitution. Even after additional attempts, with extended incubation times for the plaque assays, it was not possible to generate virus with this mutation (Fig. 1). However, A/New Jersey/8/76 (H1N1) and N1 swine viruses contain a threonine at this position (12a, 18). To see if the threonine replacement is generally accepted in the NA molecule, we performed reverse genetics to generate a virus (CYTPro-Thr) containing the Pro-to-Thr mutation. This attempt was successful (Fig. 1). Sequence analysis of the transfectant virus did not reveal any additional mutations in the NA molecule. The tissue culture infectivity (6.7 $\log_{10}$ of PFU/ml) was lower than that of the wild-type virus (8.0 $\log_{10}$ of PFU/ml), and the plaque size was smaller (Fig. 1), indicating that this mutation affects virus replication. These results show that virus replication does not require a conserved proline residue but is affected by structural changes at this position.

**Incorporation of mutant NA molecules into virions.** Mutations in the NA cytoplasmic tail affect incorporation of the protein into virions (2). To determine the effect of a tail deletion, we quantified the amount of viral glycoproteins in purified viruses by growing the transfectant viruses in MDBK cells in the presence of [$^3$H]mannose. The amount of NA was normalized to the amount of hemagglutinin (HA) on the virions after separating the viral glycoproteins by SDS-PAGE as described in Materials and Methods.

Deletion of the cytoplasmic tail drastically reduced NA incorporation into virions (Fig. 2), suggesting that this region contains residues vital to the incorporation signal. NOTAIL/4AE and NOTAIL/7CC had only 14 and 59% as much NA incorporated into their respective progeny virions as did the wild-type virus. The greater proportion incorporated into NOTAIL/7CC may indicate compensation by the transmembrane mutation for the deleted cytoplasmic tail. The Pro-to-Thr mutation (CYTPro-Thr) also affected NA incorporation (23% reduction), but not as extensively as the changes in the NOTAIL mutants (Fig. 2).

**Cell surface expression of NA cytoplasmic tail mutants.** The reduced levels of NA incorporated into the virions may have resulted from decreased expression of this protein on the cell surface. We examined this possibility by FACS analysis. Cells were infected with virus, and at different times postinfection, suspended cells were divided into two aliquots, one labeled with an anti-NA monoclonal antibody and the other labeled with a pool of anti-HA monoclonal antibodies. The amount of NA expressed on the cell surface was normalized to the amount of HA expressed, after calculation of the mean fluorescence of both reagents (Fig. 3).

For all viruses, including the wild type, NA staining identified two positive populations of cells that differed in fluorescence intensity for unknown reasons, whereas HA staining detected a single positive population at 6 and 8 h and two populations only at 12 h postinfection (Fig. 3). Since all samples contained two cell populations, neither of which could be considered irrelevant, we included both populations in all subsequent calculations. Fluorescence analysis of the NA staining results (normalized to the value for HA) showed that all mutant NA proteins were expressed on the cell surface at the same levels as the wild-type NA, assuming that the mutation in the NA cytoplasmic domain did not affect HA incorporation. At 6 h postinfection, the HA/NA fluorescence intensity ratio on the cell surface was approximately 10:1 for mutant and wild-type viruses (Fig. 4). Between 6 and 12 h postinfection, the kinetics of NA expression for the mutants were similar to that of the wild-type virus, although at 12 h postinfection, a 25 to 30% decrease in NA expression for NOTAIL/4AE and CYTPro-Thr was observed. Although electron microscopic analysis of cells infected with NA tail mutants showed that progeny virions form large aggregates on the cell surface (see below), similar levels of expression of HA and NA were obtained even after bacterial neuraminidase treatment of infected cells. This treatment dissociates the aggregated virions from the cell surface, thus eliminating the possibility that aggregated virions contribute significantly to the level of cell surface HA and NA expression determined by FACS analysis (data not shown). These findings indicate that limited cell surface expression of NA does not contribute to the reduced levels of the molecule's incorporation into virions.

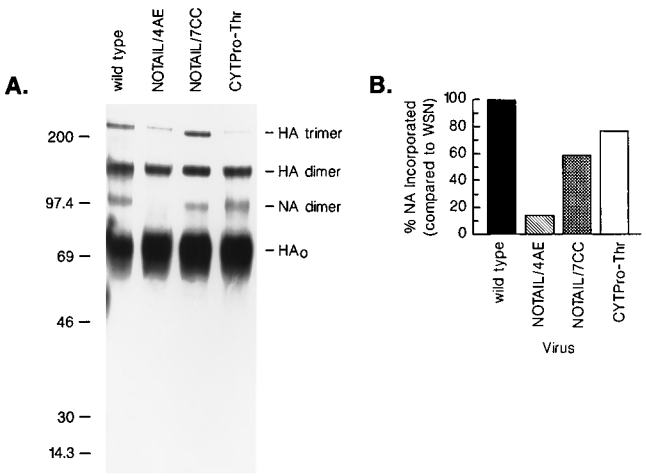


FIG. 2. Reduction of NA incorporation into NOTAIL virions. (A) NA cytoplasmic tail mutants were grown in MDBK cells in the presence of [$^3$H]mannose. Purified virus was disrupted in lysis buffer and analyzed by nonreducing SDS-PAGE. Because of differences in the extent of [$^3$H]mannose labeling of HA and NA on the virion (2), gels were exposed for 1 day and 21 days. The autoradiograph shown was exposed for 21 days. Positions of molecular weight markers are indicated in kilodaltons on the left. $HA_0$, uncleaved HA. (B) The percentage of NA incorporated into virions relative to the wild-type level. The HA/NA ratios were determined by scanning densitometry of autoradiographs. To ensure that the band intensities on autoradiographs were in the linear range, we derived HA values from 1-day exposures and NA values from 21-day exposures. The reported values are based on a wild-type HA/NA ratio of 100%.

**NA activities.** Reduced levels of NA incorporation into the virions should lead to lower enzymatic activity per virion. This prediction was tested by incubating purified viruses with 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid substrate and determining the NA activity from the amount of a fluorescent compound, 4-MU, produced (26). NA specific activity

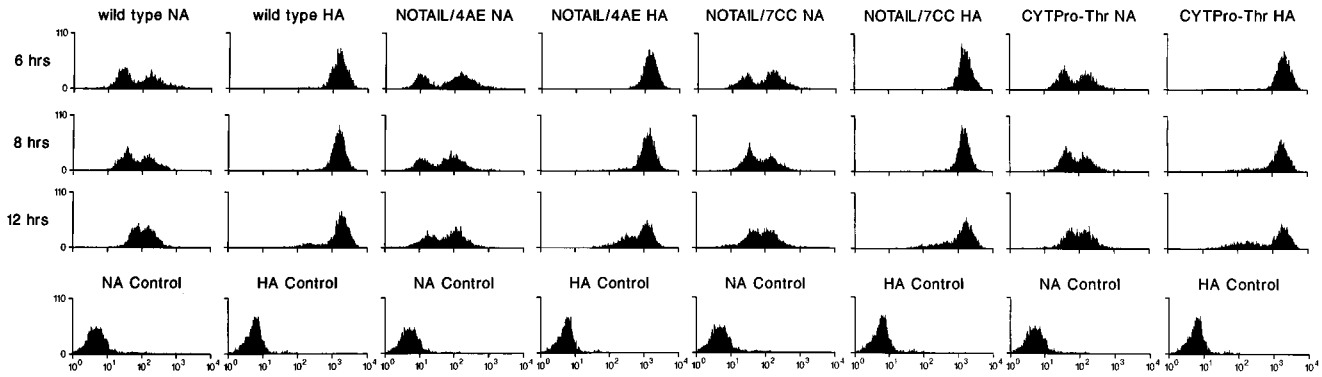


FIG. 3. Comparison of the amounts of HA and NA glycoproteins expressed on the surface of MDCK cells. At 6, 8, and 12 h postinfection (multiplicity of infection of 3), MDCK cells expressing HA and NA glycoproteins were analyzed by FACS cytometry. The profiles shown depict cell number as a function of log fluorescence intensity of HA and NA expression for wild-type virus, NOTAIL/4AE, NOTAIL/7CC, and CYTPro-Thr. NA and HA control samples were taken at 12-h time points.

was calculated as micromoles of 4-MU per microgram of viral protein (Fig. 5).

The mutant viruses consistently showed less NA specific activity than did the wild-type virus after 60 min: 88% less for NOTAIL/4AE, 52% less for NOTAIL/7CC, and 62% less for CYTPro-Thr. The higher activity of NOTAIL/7CC than of NOTAIL/4AE correlates with the higher amount of NA incorporated into the virions (Fig. 2). The reduction of NA activity in CYTPro-Thr compared with NOTAIL/7CC, together with a greater incorporation of CYTPro-Thr-NA molecules in virions, suggests that substitution of the conserved proline residue with threonine may directly affect NA activity by altering its enzymatically active conformation.

**Aggregation and morphologic changes of mutant viruses on infected cultured cells.** Progeny influenza viruses having little or no NA enzymatic activity form large aggregates on the cell surface (17, 21), because NA activity is needed to remove sialic acid from the viral and cellular glycoconjugates (1a, 15, 21). Thus, in accord with their significantly reduced levels of NA proteins, the NOTAIL mutants (but not the wild-type virus) produced large aggregates of progeny virus on the cell surface, as revealed by electron microscopy (Fig. 6). The NOTAIL mutant virions also appeared to be long and filamentous, as opposed to the spherical virions commonly seen among wild-type viruses. Neither the loss of NA enzymatic activity nor aggregation of the progeny particles at the cell surface appears to contribute to the morphologic change in virions, as disaggregation of virions with purified bacterial neuraminidase did not affect the formation of filamentous particles (Fig. 6).

To investigate this morphologic difference more closely, we examined both purified NOTAIL and wild-type viruses in the electron microscope by using the negative-staining method (19). As shown in Fig. 7, filamentous particles were typically seen in preparations of mutant viruses, but they were exceedingly rare in wild-type virus preparations. Analysis of different micrographic fields revealed that approximately 20% ($n = 100$) of the NOTAIL/4AE and 15% ($n = 100$) of the NOTAIL/7CC virions were filamentous, compared with only 1% ($n = 100$) of the wild-type virions. A particle was considered filamentous if its length was more than three times its diameter. Because the circular images observed were counted as spherical particles and not as cross-sections of filamentous particles, this calculation underestimates the true frequency of the latter. The frequencies of filamentous particles observed with negative staining were lower than those seen during virus budding, when more than 50% of the NOTAIL virions were filamentous (Fig. 6). This apparent discrepancy may reflect the disruption of filamentous particles during the purification procedure needed for negative staining.

Differences in viral morphology could be related to sequence variations in the matrix (M) gene, as shown by others (24).

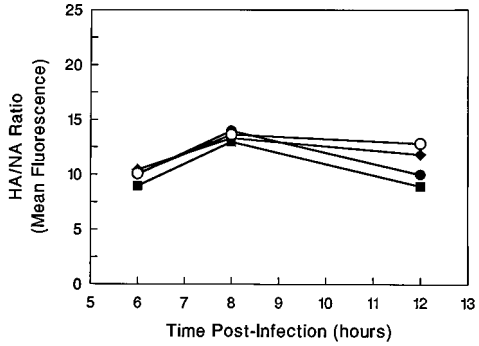


FIG. 4. Expression of viral glycoproteins on the surface of MDCK cells. At the indicated times postinfection, the HA/NA ratios were calculated from the mean fluorescence intensities determined in Fig. 3. ○, wild type; ◆, NOTAIL/7CC; ●, CYTPro-Thr; ■, NOTAIL/4AE.

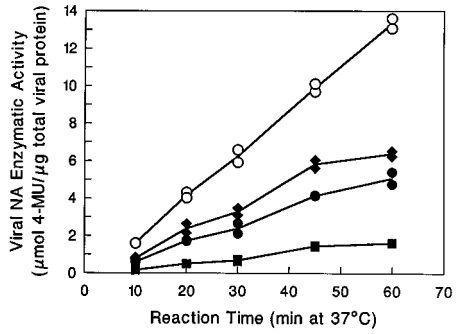


FIG. 5. Reduced virion NA activities of cytoplasmic tail mutants. Identical amounts of purified virus (0.5 μg of total viral protein) were incubated (37°C), in duplicate reactions, with 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid for the indicated times (total reaction volume of 10 μl). The fluorescence of release of 4-MU was determined ($\lambda_{ex} = 355$ nm; $\lambda_{em} = 460$ nm) after reactions were stopped by addition of 200 μl 0.1 M glycine buffer (pH 10.7) containing 25% (vol/vol) ethanol. ○, wild type; ◆, NOTAIL/7CC; ●, CYTPro-Thr; ■, NOTAIL/4AE.

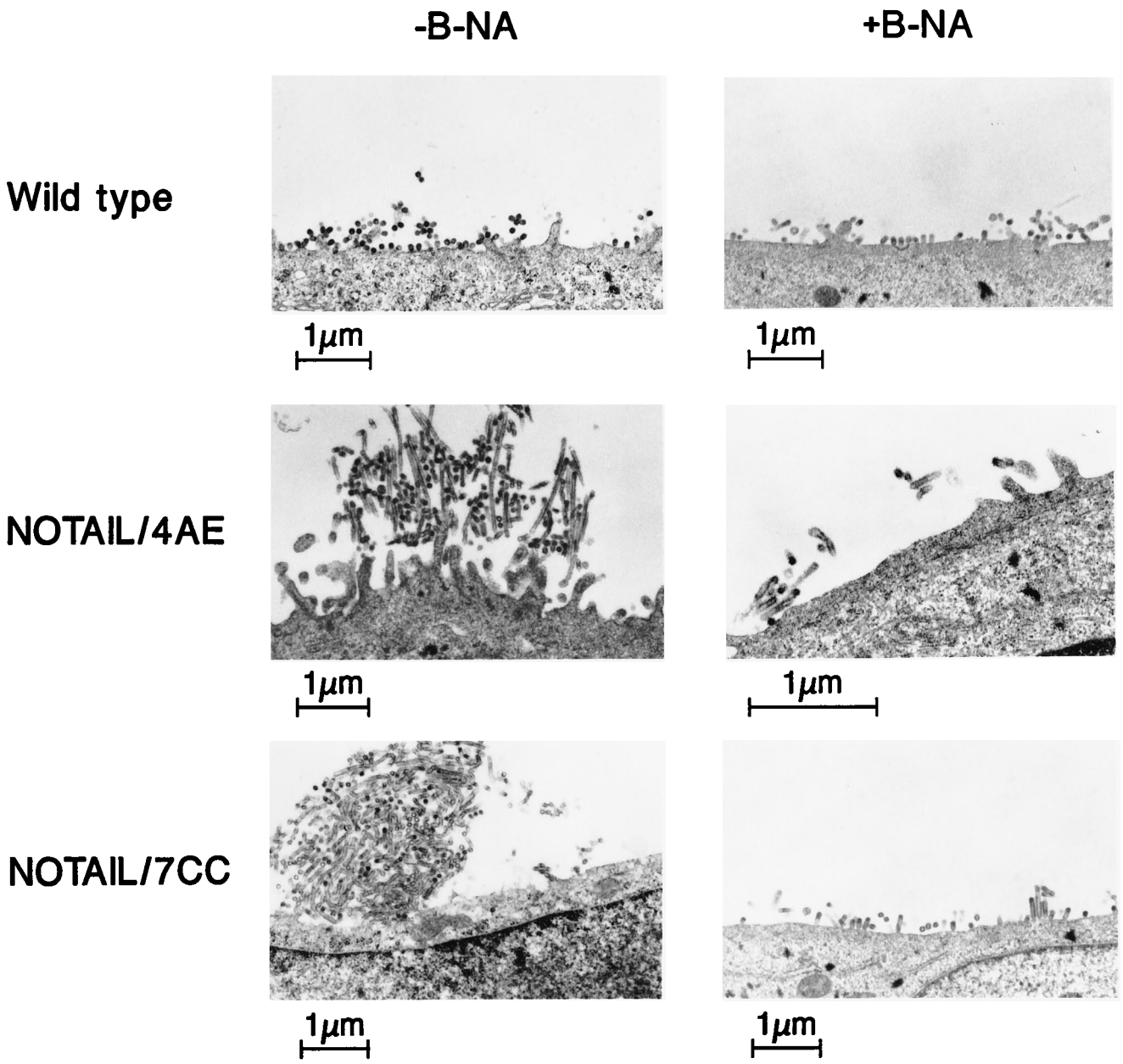


FIG. 6. Aggregation of NOTAIL mutant viruses on the cell surface. MDCK cells were infected with either wild-type virus or NOTAIL/4AE or NOTAIL/7CC mutant virus. At 10 h postinfection, the cells were processed for electron microscopy as described in Materials and Methods (−B-NA) or treated for 1 h at 37°C with 5.5 mU of purified *V. cholerae* neuraminidase and then processed (+B-NA).

However, comparison of M gene sequences between the NOTAIL mutants and wild-type viruses failed to reveal any mutations, suggesting that the NA cytoplasmic tail plays a role in the formation of virus particles and implying an interaction of this region with other proteins (viral or cellular).

**Virulence in mice.** The growth differences observed in tissue culture among the NOTAIL mutants and wild-type virus (Fig. 1) suggested that the defining mutations may alter infectivity in vivo. When tested by intranasal infection of mice, the wild-type virus was highly virulent, whereas both the NOTAIL/4AE and NOTAIL/7CC mutants were attenuated (Fig. 1). The mechanism of attenuation appears to involve reduced levels of NA incorporation into virions, resulting in lower NA enzymatic activity, which results in the aggregation of progeny viruses on the cell surface (Fig. 6) and the consequent inability of the virus to be released.

## DISCUSSION

In this report, we demonstrate that the conserved residues of the NA cytoplasmic tail are not absolutely essential for influenza A virus replication but do influence virion formation, acting as part of the incorporation signal of NA molecules into progeny virions. Similar incorporation signals have been identified in the transmembrane and cytoplasmic domains of the influenza virus HA glycoprotein (20). Garcia-Sastre and col-

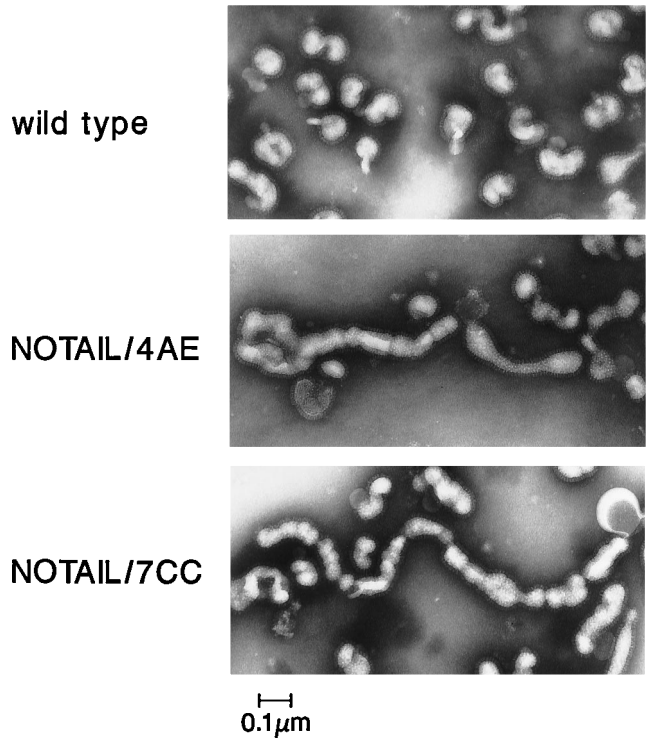


FIG. 7. Negative staining of purified viruses. Purified virions were negatively stained with 2% phosphotungstic acid (pH 6.4) and examined with a Philips 301 electron microscope.

leagues extended this finding by showing that the HA transmembrane and cytoplasmic domains contain all of the signals required for incorporation of a foreign protein into the influenza virus envelope (9). However, a mutant influenza virus that lacks the HA cytoplasmic tail grows almost as well as the wild-type virus (12). Thus, the cytoplasmic domain of influenza virus glycoproteins appears to be required for efficient incorporation of viral proteins into budding virions but not essential for virus replication. Similar requirements for the cytoplasmic domain of glycoproteins of other viruses have been observed in the production of infectious virus (8, 27). This mechanism may have evolved because of the paucity of host cell glycoproteins in viral particles and the need to distinguish between viral and cellular proteins during virion assembly.

Recently, Liu and Air (16) generated an NA-deficient virus (NWS-Mvi) that contains a truncated NA gene with a large internal deletion of 800 to 900 nucleotides, leaving regions encoding the cytoplasmic and transmembrane domains and part of the stalk, but does not have NA enzymatic activity. It was shown that NA enzymatic activity was not needed for influenza virus entry, replication, or assembly, either in tissue culture or in a mouse model (17). Whether the truncated protein was synthesized in NWS-Mvi-infected cells is not known, but it was detected on the surface of cells after expression in the vaccinia system (1), suggesting that it may have played a role in virus replication. If the truncated NA is necessary for virus replication, our rescue of the NOTAIL mutant would shift emphasis to the role of the transmembrane domain. Alternatively, the NA molecule is not required for influenza virus replication.

Fujiyoshi et al. (7) recently showed by electron cryomicroscopy that infectious influenza virus envelopes consist of a thin, outer single phospholipid layer and an inner thick layer. From a biochemical analysis, the authors speculated that the inner layer might be modified by the M1 protein. Because the NA of the NOTAIL/7CC mutant with the Ser-12-to-Pro mutation was incorporated into virions more efficiently than the NA of NOTAIL/4AE, we suggest that the transmembrane region may interact with the M1 protein in the viral envelope. Although an NA-M1 interaction has not been directly demonstrated, it may be necessary for efficient incorporation of NA molecules into budding virions.

Although all influenza viruses are considered to be pleomorphic, some strains contain more filamentous particles than others (11). The wild-type strain used in this study, A/WSN/33, is largely spherical; however, a deletion of the cytoplasmic tail of the NA molecule (NOTAIL) led to a higher frequency of filamentous particles (Fig. 6 and 7). The lack of NA enzymatic activity does not appear to influence viral morphology, since the addition of purified bacterial neuraminidase to cells infected with NOTAIL mutant viruses still resulted in the formation of filamentous particles (Fig. 6). Since sequence analysis did not reveal mutations in the NOTAIL M gene, which is known to affect viral morphology (24), we attribute the shift from spherical to filamentous particles to deletion of the NA cytoplasmic tail and further suggest its interaction with viral and/or cellular proteins. Deletion of the HA cytoplasmic tail did not affect viral morphology in a recent study by Jin et al. (12).

Recently, García-Sastre and Palese (10) generated a NOTAIL NA mutant virus [NA/TAIL (−)] that is identical to our NOTAIL NA virus except that it contains 12 nucleotide insertions between the noncoding region and the initiation codon in the NA gene, shows reduced NA incorporation into virions (50% of the wild-type level), and replicates only 1 $\log_{10}$ lower than the wild-type virus. Because cells infected with the mutant virus contained only 50% of the NA molecules found in cells infected with wild-type virus, these investigators concluded that the signal for packaging NA into progeny virions is not located in the cytoplasmic domain. Although the precise basis for the discrepancy between their findings and ours is unclear, the inserted sequence upstream of the initiation codon may have affected NA expression in their cells. Alternatively, there might be compensatory mutations in the NA gene in their virus, since only 115 nucleotides were sequenced.

Since the NA cytoplasmic tail seems to act as part of an incorporation signal and is important for virus replication, it might be a suitable target for antiviral therapeutics. With reverse genetics already established for the NA gene, one could also consider the introduction of mutations into the NA of currently circulating viruses as an option for the production of live attenuated influenza virus vaccines.

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