Palmitoylation of the Influenza Virus Hemagglutinin (H3) Is Not Essential for Virus Assembly or Infectivity

HONG JIN,^{1,2} KANTA SUBBARAO,³ SANGEETA BAGAI,^{1,2} GEORGE P. LESER,² BRIAN R. MURPHY,³
AND ROBERT A. LAMB^{1,2*}

Howard Hughes Medical Institute¹ and Department of Biochemistry, Molecular Biology and Cell Biology,² Northwestern University, Evanston, Illinois 60208-3500, and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892-0720³

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The C terminus of the influenza virus hemagglutinin (HA) contains three cysteine residues that are highly conserved among HA subtypes, two in the cytoplasmic tail and one in the transmembrane domain. All of these C-terminal cysteine residues are modified by the covalent addition of palmitic acid through a thio-ether linkage. To investigate the role of HA palmitoylation in virus assembly, we used reverse genetics techniques to introduce substitutions and deletions that affected the three conserved cysteine residues into the H3 subtype HA. The rescued viruses contained the HA of subtype H3 (A/Udorn/72) in a subtype H1 helper virus (A/WSN/33) background. Rescued viruses which do not contain a site for palmitoylation (by residue substitution or substitution combined with deletion of the cytoplasmic tail) were obtained. Rescued virions had a normal polypeptide composition. Analysis of the kinetics of HA low-pH-induced fusion of the mutants showed no major change from that of virus with wild-type (wt) HA. The PFU/HA ratio of the rescued viruses grown in eggs ranged from that of virus with wt HA to 16-fold lower levels, whereas the PFU/HA ratio of the rescued viruses grown in MDCK cells varied only 2-fold from that of virus with wt HA. However, except for one rescued mutant virus (CAC), the mutant viruses were attenuated in mice, as indicated by a ≥ 400 -fold increase in the 50% lethal dose. Interestingly, except for one mutant virus (CAC), all of the rescued mutant viruses were restricted for replication in the upper respiratory tract but much less restricted in the lungs. Thus, the HA cytoplasmic tail may play a very important role in the generation of virus that can replicate in multiple cell types.

The influenza A virus envelope contains two major spike glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and, in much less abundance, the M₂ ion channel. The influenza virus HA glycoprotein plays three major roles in virus infection: (i) HA binds to a sialic acid-containing receptor on the cell surface, (ii) HA is responsible for penetration of the virus into the cell cytoplasm by mediating the fusion of the membrane of the endocytosed virus particle with the endosomal membrane, and (iii) HA is the major antigenic determinant of the virus against which neutralizing antibodies are produced. The HA spike glycoprotein is a homotrimer extending 135 Å (13.5 nm) from the membrane and is composed of two predominant regions: (i) a long fibrous stem, extending 76 Å (7.6 nm) from the membrane, which forms a triple-stranded coiled coil of α -helices and (ii) the globular head of the molecule, which contains the sialic acid-binding pocket and most of the antigenic epitopes (reviewed in references 13 and 34).

The HA cytoplasmic tail contains 10 or 11 residues, under the assumption that the charged residue adjacent to the hydrophobic TM region delineates the boundary of the domain. The HA cytoplasmic tail is highly conserved among the 14 HA subtypes (19, 33). Three conserved cysteine residues (amino acids 555, 562, and 565), one located in the TM domain and two located in the cytoplasmic tail, are modified by the covalent linkage of palmitate through a thio-ether linkage (16, 17, 23, 31, 32). Analysis of cysteine substitution mutants initially indicated that palmitoylation of HA was essential for HA fusion

activity (16). However, many other analogous experiments did not support these findings (17, 29, 31, 32).

Although the HA cytoplasmic tail is not essential for intracellular transport of HA (4), the presence of a conserved sequence in this domain made it seem likely that the cytoplasmic tail of HA would be involved in forming essential protein-protein interactions in virus assembly. However, it was found, using a complementation assay, that whereas recombinant HA molecules with heterologous tails were excluded from virions, HA lacking a cytoplasmic tail was assembled into virus particles (18). Furthermore, it was found to be possible to generate an influenza virus that contained an HA devoid of a cytoplasmic tail (tail⁻ HA virus) (10), by using a reverse genetics procedure that makes it possible to introduce a cDNA-derived RNA gene segment into an infectious influenza virus genome (14). The tail⁻ HA virus assembled and replicated almost as efficiently as virus with wild-type (wt) HA (wt HA virus), suggesting that the cytoplasmic tail is not necessary for the virus assembly process. Nonetheless, a revertant virus was isolated, suggesting that possession of an HA cytoplasmic tail does confer an advantage (10).

Influenza virus genes in the absence of selective pressure do not maintain sequence conservation. Thus, the failure to find an essential biological role for palmitoylation of the cytoplasmic tail when HA is expressed from cDNA or to find an obligate requirement for the cytoplasmic tail in rescued influenza virus creates a conundrum. Thus, it was of considerable interest that when mutations were introduced into the conserved C-terminal and palmitoylated cysteine residues of the A/WSN/33 HA (H1 subtype) by the reverse genetics technique, mutations at the equivalent of cysteine residue 562 led to highly attenuated viruses and mutations at the equivalent of cysteine residue 565 failed to yield infectious-particle formation (38). From these

* Corresponding author. Mailing address: Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 North Campus Dr., Evanston, IL 60208-3500. Phone: (708) 491-5433. Fax: (708) 491-2467. Electronic mail address: ralamb@nwu.edu.

FIG. 1. Schematic diagram of wt HA and HA cytoplasmic tail mutations. The expanded region on the upper line shows the nucleotide and amino acid sequences of the HA cytoplasmic tail and its junction with the transmembrane (TM) domain. The HA cytoplasmic tails of tail⁻ HA and Mtail⁻ HA were deleted by insertion of one or three translational stop codons, respectively. The nomenclature for the rescued transfectant viruses used in this paper is shown in the right column.

data, it was suggested that palmitylation has a functional role in virus assembly, although it could not be excluded that the introduced substitutions had a dominant negative effect on virus assembly (38). Since we had found it possible to delete the cytoplasmic tail of the A/Udorn/72 HA (H3 subtype) and generate the tail⁻ HA virus (10), it was important to examine the roles of the conserved C-terminal and palmitylated cysteine residues in the H3 subtype HA in the context of infectious virus. Here we report properties of rescued influenza viruses containing substitutions at each of the three cysteine residues.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells, Madin-Darby canine kidney (MDCK) cells, and CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum. Influenza virus A/WSN/33 was propagated in MDBK cells, and influenza virus A/PR/8/34 was grown in 11-day embryonated chicken eggs. Ribonucleoprotein transfections were performed with MDBK cells, and biological cloning of viruses by harvesting plaques was performed with MDCK cells.

Oligonucleotide-directed mutagenesis of A/Udorn/72 HA cDNA. Plasmid pT7HA contains the full-length cDNA of the wt HA gene of influenza virus A/Udorn/72 (10) flanked by a bacteriophage T7 RNA polymerase promoter at one end such that RNA transcripts can be made in the viral RNA sense. At the other end of the plasmid is an *EatI* site which provides a unique and precise site for linearization of the template, and it was used for the in vitro synthesis of the RNA transcripts. Plasmid pT7HA was used for the construction of the HA cytoplasmic tail mutations. The A/Udorn/72 HA cytoplasmic tail cysteine replacement mutants CAC, CCP, and CAY (Fig. 1) described previously (29) were altered for cloning manipulations by PCR, and the *Bam*HI-to-*Pst*I fragment that encodes the HA₂ C-terminal region was subcloned into pT7HA. The mutation CCA was made by oligonucleotide-directed mutagenesis of the plasmid pT7HA by using a four-primer PCR method. To construct Mtail⁻ HA, three stop codons were introduced at the boundary of the HA transmembrane domain and the cytoplasmic tail, and cysteine residue 555 in the transmembrane domain was replaced with methionine by using PCR. The MAY mutation was synthesized by PCR with CAY as a template. The nucleotide sequences of the *Bam*HI-to-*Pst*I cDNA fragments which had been subcloned into pT7HA and encode the HA₂ C-terminal regions were determined (25). Recombinant DNA procedures were performed by standard PCR and cloning techniques (1). Sequencing of influenza virus RNA by reverse transcription and asymmetric PCR sequencing was done as described previously (10).

Rescue of transfectant viruses containing A/Udorn/72 HA RNA segment. Influenza virus nucleoprotein (NP) and the three polymerase proteins were purified from influenza virus A/PR/8/34 as described previously (20) and were used to encapsidate HA RNA transcribed in vitro by T7 RNA polymerase essentially as described by Enami and Palese (5). The protocol used for introducing

A/Udorn/72 HA RNA into A/WSN/33 helper virus has been described previously (10); this protocol uses the general principles established previously (5, 14, 20).

Virus purification and peptide N-glycosidase F digestions. The wt and mutant transfectant viruses were propagated in embryonated eggs and purified by centrifugation on 20 to 60% sucrose density gradients. For peptide N-glycosidase F digestion, 30 µg of viral proteins was treated with 0.5% sodium dodecyl sulfate (SDS)-0.5% Nonidet P-40-20 mM Na₂HPO₄ and digested for 20 h with 1 U of peptide N-glycosidase F (Boehringer-Mannheim, Indianapolis, Ind.) in the presence of protease inhibitors as described previously (22).

Metabolic labeling of infected cells, immunoprecipitation, and SDS-PAGE. Monolayers of CV-1 cells were infected with wt influenza virus and transfectant influenza viruses at a multiplicity of infection of 5 to 10. Cultures at 4 h postinfection (p.i.) were incubated in DME deficient in cysteine for 30 min and then labeled with [³⁵S]cysteine (100 µCi/ml) in DME deficient in cysteine for 60 min. Metabolic labeling of virus-infected cells with [9,10(n)-³H]palmitic acid was performed as described previously (6). Briefly, virus-infected cells at 3 h p.i. were labeled with [³H]palmitic acid (0.5 mCi/ml) in DME supplemented with 10% tryptose phosphate, 1 mM Na pyruvate, and 1% dimethyl sulfoxide for 4 h. Cleavage of HA₀ to HA₁ and HA₂ by TPCK [L-tosylamido-2-phenyl]ethyl chloromethyl ketone-treated trypsin (TPCK-trypsin) (Worthington Biochemical, Inc., Freehold, N.J.), immunoprecipitation of proteins, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were done as described previously (10). Goat polyclonal antiserum to A/Udorn/72 HA (SP31) and mouse monoclonal antibody 14C2 to influenza virus M₂ protein were used (36).

Membrane fusion assays. Labeling of virions with octadecyl rhodamine B chloride (R18) (Molecular Probes, Eugene, Oreg.) was performed essentially as described previously (2). The fluorescent probe R18 was inserted into the viral bilayer by injecting 10 µl of a 1-mg/ml R18 solution in ethanol into 300 µg of virions in phosphate-buffered saline (PBS) (1 mg/ml) with vigorous vortexing. After incubation with constant shaking for 1 h at room temperature, the unbound probe was removed by centrifugation at 45,000 rpm for 30 min in a Beckman TLA55 rotor. The R18-labeled virus pellet was washed with PBS by centrifugation, and 20 µg of R18-labeled virus was used to bind to a suspension of 5 × 10⁶ MDCK cells at 4°C for 60 min. The unbound virions were removed by washing three times with PBS. An aliquot of the R18-labeled virus-MDCK complex was placed in a thermostable cuvette containing 3 ml of PBS prewarmed to 37°C. Fusion was initiated by adding the predetermined amount of 0.25 M citric acid to lower the solution to pH 5.0. Fluorescence changes as a result of fusion of R18-labeled virus-cell complexes were measured continuously with a spectrofluorimeter (Alpha Scan; Photon Research Inc., South Brunswick, N.J.) with a 1-s time resolution at excitation and emission wavelengths of 560 and 590 nm, respectively. A 570-nm-cutoff filter was placed in the emission optical pathway to reduce scattering. The samples were then adjusted to 0.1% Triton X-100 to obtain the amount of fluorescence at infinite dilution. The percent fluorescence dequenching (% FDQ) at any time point was calculated by using the equation % FDQ = [(F - F₀)/(F_i - F₀)] × 100, where F equals the fluorescence intensity at a specified time, F₀ is the fluorescence before the pH was lowered, and F_i is the fluorescence at infinite dilution. Three aliquots from each sample were independently measured. Cytoplasmic content mixing of virus-infected

CV-1 cells with human erythrocytes preloaded with NBD-taurine was performed as described previously (26).

Infection of transfectant influenza viruses in mice. Five-week-old female outbred NIH Swiss mice anesthetized with methoxyflurane were inoculated intranasally with 0.05 ml of virus. To determine the 50% lethal dose (LD_{50}), groups of six mice were inoculated with 2.0, 3.0, 4.0, or 5.0 $\log_{10}$ PFU of the transfectant viruses and were observed daily for 20 days. Deaths were recorded, and the LD_{50} s were calculated by the Karber method (15). To determine the level of virus replication in the respiratory tract, mice were sacrificed on day 3 or 4 by exposure to carbon dioxide, and their nasal turbinates and lungs were removed for virus quantification as described previously (30). Virus titers were expressed as $\log_{10}$ 50% tissue culture infective doses per gram.

RESULTS

Generation of mutations in the C-terminal region of influenza virus HA₂. To examine the roles of the three conserved cysteines (residues 555, 562, and 565) in the C-terminal transmembrane-cytoplasmic tail region of influenza virus HA₂, mutations were introduced into a cDNA copy of the A/Udorn/72 HA. The nomenclature for the mutants defines the amino acids, in order, found at residues 555, 562, and 565; e.g., HA containing the changes C-555 to M, C-562 to A, and C-565 to Y is designated MAY. In addition to these point mutations, we used a mutant described previously, tail⁻ HA (10), which contains a translational stop codon at the presumptive junction of the transmembrane domain and cytoplasmic tail (replacing residue 557). Furthermore, we constructed a mutant, Mtail⁻ HA, which contains methionine substituted for cysteine 555 and contains three translational stop codons replacing residues 557, 558, and 559. The rationale for introduction of a methionine residue in Mtail⁻ HA in place of cysteine 555 was that the H8 and H9 influenza A virus subtypes contain this substitution naturally (19), and thus we thought it less likely that this change would be deleterious to HA. Mtail⁻ HA contained three consecutive translational stop codons to lessen the likelihood of obtaining revertant rescued viruses, since we observed previously that revertants of transfectant tail⁻ HA virus could be readily isolated (10). Each of the seven HA molecules oligomerized to form trimers, and examination of the intracellular transport kinetics and cell surface expression of HA indicated that CAC, CCA, CCP, CAY, and MAY HA molecules were transported to the cell surface with kinetics comparable to those of wt HA (half-life ~ 45 min), and tail⁻ HA and Mtail⁻ HA were delayed equivalently (half-life ~ 65 min) (29) (data for MAY HA and Mtail⁻ HA are from unpublished observations).

Rescue of influenza viruses containing HA₂ molecules with altered C-terminal domains. In an attempt to generate rescued (transfectant) influenza viruses containing the HA cysteine substitution mutations and the Mtail⁻ HA mutation, HA RNA-ribonucleoprotein complexes were reconstituted in vitro and transfected into cells infected with influenza virus A/WSN/33. To select for rescued viruses containing the H3 HA (A/Udorn/72), cells were incubated with a pool of monoclonal antibodies to A/WSN/33 HA, and viruses released into the culture medium were biologically cloned by plaque assay. The nature of the HA introduced into rescued viruses was examined by immunoprecipitation of virus-infected cell lysates with antiserum specific for A/Udorn/72 HA, and the presence of mutations was confirmed by nucleotide sequence analysis of virion RNA segment 4 (data not shown). Transfectant viruses containing each of the mutations shown in Fig. 1 were obtained. The virus referred to as wt HA transfectant virus contains the A/Udorn/72 HA introduced into the A/WSN/33 helper virus genetic background.

The polypeptide compositions of egg-grown transfectant viruses were analyzed by SDS-PAGE, and polypeptides were stained with Coomassie brilliant blue (Fig. 2A). Both tail⁻

HA₂ and Mtail⁻ HA₂ species were observed to migrate significantly faster than wt HA₂ (Fig. 2). To attempt to resolve the NP-HA₁-NA band into individual components, prior to gel analysis virions were incubated with peptide N-glycosidase F to remove carbohydrate chains from the glycoproteins (Fig. 2B). The identities of deglycosylated NA and HA species were confirmed by immunoblotting analysis (data not shown). For HA₂, the digestion did not go to completion, and both glycosylated and deglycosylated species are indicated (Fig. 2B). To examine the relative abundances of the identifiable virion polypeptide species in the transfectant viruses, the polypeptide staining patterns (Fig. 2B) were quantified by densitometry, and the staining for each transfectant virus was standardized to the staining of NP. All of the transfectant viruses contained approximately equivalent amounts of HA, NA, and matrix protein (M₁) relative to that of NP (Table 1), although Mtail⁻ on occasion contained more HA than did wt HA virus. The amount of M₂ protein found in virions is too small to be readily detected by staining (36). However, as the virion-associated M₂ protein has been postulated to be involved in viral assembly (3, 9, 36, 37), in addition to its ion channel activity, the relative amounts of M₂ found in the transfectant viruses containing altered HA cytoplasmic tails and wt HA virus were compared by immunoblotting of the M₂ protein and probing with the M₂-specific 14C2 monoclonal antibody. Under the conditions used, the intensity of the chemiluminescence signal for M₂ protein is linear with respect to the amount of protein present (8). Immunoblotting of HA was also performed for comparison with that of M₂ protein. Densitometer tracing of the X-ray film, normalization to the relative intensity of the HA signal, and adjustment for the HA/NP ratio (with the amount of NP set at 1) indicated that the amount of M₂ protein varied approximately two- to threefold among transfectant viruses compared with HA.

Incorporation of [³H]palmitic acid into HAs of rescued transfectant viruses. It has been shown previously, largely through mutagenesis studies and expression of altered HAs from cDNAs, that the three C-terminal proximal cysteine residues in HAs of the H2, H3, and H7 subtypes (for the H3 subtype, cysteine residues 555, 562, and 565) are modified by the covalent addition of palmitate through a thio-ether linkage (16, 17, 27, 31, 32). To determine whether the HA cytoplasmic tail cysteine-altered and tail⁻ HA transfectant viruses were modified by palmitylation, virus-infected cells were metabolically labeled with [³⁵S]cysteine or [³H]palmitic acid, and HA was immunoprecipitated from untreated lysates (Fig. 3A) or from lysates treated with trypsin to cleave HA₀ to HA₁ and HA₂ (Fig. 3B). For comparison, the vesicular stomatitis virus G protein, which is known to be modified by a single palmitate moiety (24, 28), was labeled in parallel. As shown in Fig. 3, the Mtail⁻ HA and MAY transfectant viruses, both of which lack the three C-terminal proximal cysteine residues, could not be labeled with [³H]palmitic acid. In comparison, the HA₂ polypeptides of transfectant viruses which contain a C-terminal proximal cysteine residue could be labeled with [³H]palmitic acid. The HA₂ doublet observed in CAY and MAY transfectant virus-infected cells (Fig. 3B, [³⁵S]cysteine labeled) is thought to be due to partial proteolysis of the altered C terminus. The relative ratios of [³H]palmitate to [³⁵S]cysteine were compared by densitometry of autoradiograms for each transfectant in five separate labeling experiments. However, for each transfectant the ratio was found to vary twofold between experiments, possibly because the palmitate pools in the cells were not saturated. However, in a single experiment, as shown in Fig. 3, it can be seen that the specific amino acid substituted for cysteine 565 appears to affect the degree of

FIG. 2. Polypeptide compositions of transfectant viruses containing HA cytoplasmic tail mutations. The indicated viruses were grown in embryonated eggs and purified through sucrose gradients. The nomenclature for the transfectant viruses is as in Fig. 1. UD, A/Udorn/72 virus. (A) Viral polypeptides were separated by SDS-15% PAGE and stained with Coomassie brilliant blue. Positions of molecular weight markers (in thousands) are shown. (B) Purified virions were solubilized in 0.5% SDS-0.5% Nonidet P-40 and digested with peptide N-glycosidase F to remove carbohydrate chains from HA and NA. Polypeptides were stained with Coomassie brilliant blue. Note that both tail⁻ HA₂ and Mtail⁻ HA₂ have faster mobilities than wt HA₂ and that peptide N-glycosidase F digestion did not go to completion: both glycosylated and deglycosylated HA₂ species are indicated. (C) Immunoblot of HA₁ and M₂ of purified virions. Blots were probed with antiserum SP31, specific for A/Udorn/72 HA (21), or with antiserum 14C2, specific for the M₂ protein (36), followed by horseradish peroxidase-conjugated secondary antibody, and immune complexes were detected with the enhanced chemiluminescence system.

palmitoylation of the remaining two cysteine residues (compare lanes CCA and CCP). Previously it has been found for the H2 HA subtype that the middle cysteine (equivalent to residue 563) incorporated at least half of the fatty acid label and that the third cysteine residue distal to the HA₂ C terminus (equivalent to residue 555) incorporated only 10% of the palmitate labeling (17).

TABLE 1. Amounts of polypeptides in rescued virions normalized to that of NP

Polypeptide	Relative amt ^a in:							
	wt HA	Tail ⁻	Mtail ⁻	CAC	CCA	CCP	CAY	MAY
HA ₁	0.96	0.91	1.68	0.82	1.08	0.80	0.80	1.02
NA	0.34	0.40	0.32	0.28	0.32	0.38	0.29	0.25
M ₁	1.49	1.27	1.24	1.26	1.55	1.35	1.30	1.20

^a The Coomassie brilliant blue-stained gel shown in Fig. 2B was scanned with a densitometer, and the relative staining intensity of each polypeptide was normalized to that of NP (set at 1.00) for each transfectant virus.

Infectivity of transfectants containing HA₂ molecules with altered C-terminal domains. The infectivities of the transfectant viruses that contain HA₂ molecules with mutant C-terminal domains were determined. The transfectant viruses were plaque-to-plaque purified three times in MDCK cells and amplified in eggs. The stock viruses were then used to infect embryonated eggs or MDCK cells, and the titer of the virus yield was determined by plaque assay on MDCK cells. The plaque size of each of the transfectant viruses grown in eggs or MDCK cells was found to be similar to that of the wt HA transfectant virus. The plaque titer for the cysteine mutant transfectant viruses grown in eggs ranged from 0- to 23-fold less than that of the wt HA transfectant virus (Table 2). Another indicator of virus infectivity is to measure the ratio of the number of physical particles to the number of infectious particles. The HA titer is an appropriate measure of physical particles, as the amounts of HA found in purified virions were similar among transfectant viruses (Table 1) and analysis of the morphology of negatively stained virions in an electron microscope showed mainly spherical virions with similar distribu-

FIG. 3. [³H]palmitate labeling of HA cytoplasmic tail cysteine mutants. Influenza virus- or transfectant virus-infected CV-1 cells at 3 h p.i. were labeled with [³⁵S]cysteine or [³H]palmitic acid for 4 h. The cell extracts were untreated (A) or treated with TPCK-trypsin to cleave HA to HA₁ and HA₂ (B), proteins were immunoprecipitated with serum SP31 specific for A/Udorn/72 HA, and polypeptides were analyzed by SDS-15% PAGE. HA₂ of CAY and MAY migrated as a doublet, which is due to partial cleavage by the exogenously added trypsin (data not shown). VSV, vesicular stomatitis virus.

tions of surface spikes (reference 10 and data not shown). The PFU/HA ratio of the egg-grown transfectant virus CAC was essentially the same as that of the wt HA transfectant virus, whereas egg-grown transfectant viruses CCA, CCP, CCY, and

MAY were found to have an 8- to 16-fold lower PFU/HA ratio than the wt HA transfectant virus (Table 2). However, complete removal of the HA cytoplasmic tail caused only two- and fourfold reductions in the PFU/HA ratio for the tail⁻ HA and

TABLE 2. Infectivities of rescued viruses in embryonated eggs^a

Virus	Titer (PFU/ml [10 ⁷])	HA/ml	PFU/HA (10 ³)	PFU/HA normalized to that of wt HA
wt HA	8.0	1,024	78.10	1.0
CAC	9.0	1,024	87.89	1.125
CCA	1.0	1,024	9.76	0.125
CCP	0.5	1,024	4.88	0.062
CAY	0.6	1,024	5.86	0.075
MAY	0.35	512	6.84	0.088
Tail ⁻	2.0	512	39.06	0.50
Mtail ⁻	1.0	512	19.53	0.25

^a Embryonated chicken eggs were infected with 10³ to 10⁴ PFU of virus and incubated at 35°C for 3 days. The infectivity was determined by plaque assay in MDCK cells.

Mtail⁻ HA transfectant viruses, respectively (Table 2). To measure the titers of the transfectant viruses grown in MDCK cells, the cells were infected at a low multiplicity of infection (0.01 PFU per cell), and the plaque titer was assayed after 3 days. As shown in Table 3, it was found that both the infectivity titer and the HA titer were reduced for all transfectant viruses (including the wt HA virus) compared with viruses grown in eggs. However, the PFU/HA ratio varied only fourfold, and some of the transfectant viruses (CAC and CCP) had a PFU/HA ratio twofold higher than that of the wt HA transfectant virus.

Replication of the HA cysteine-altered transfectant viruses in mice. The WSN virus causes a lethal pneumonitis in mice following intranasal infection, permitting determination of an LD₅₀ (35). Thus, to examine whether the HA cysteine-altered transfectant viruses were attenuated *in vivo*, mice were inoculated with the transfectant viruses. As shown in Table 4, except for transfectant virus CAC, the mean LD₅₀ of each of the cysteine-altered transfectant viruses was about 10³ times higher than that of the wt HA transfectant virus. The levels of virus replication in nasal turbinates and lungs were also analyzed. Again, except for transfectant virus CAC, each of the HA cysteine-altered viruses was highly restricted in replication in the upper respiratory tract but much less restricted in the lungs. To determine if reversion of the mutations introduced into the transfectant viruses had occurred during replication in mice, the nucleotide sequence of RNA segment 4 encoding the relevant C-terminal domain of HA₂ of virus isolated from mice was determined. RNA was extracted from mice lung homogenates 4 days after infection and transcribed by reverse transcriptase, and the cDNA was amplified by PCR. Both the tail⁻ HA and Mtail⁻ HA viruses maintained the tail⁻ genotype, and

TABLE 4. Virulence and replication of HA cysteine-altered transfectant viruses in mice^a

Virus ^b	Mean log ₁₀ LD ₅₀ ^c ± SD	Virus titer (mean log ₁₀ TCID ₅₀ /g ^d ± SEM) in:			
		Nasal turbinates		Lung homogenates	
		Day 3	Day 4	Day 3	Day 4
wt HA	2.3 ± 0.3	6.5 ± 0.2	6.5 ± 0.2	7.5 ± 0.2	7.5 ± 0.1
CAC	2.8 ± 0.2	6.5 ± 0.1	6.3 ± 0.3	7.8 ± 0.2	7.5 ± 0.2
CCA	5.5 ± 0	3.8 ± 0.4	3.3 ± 0.5	6.5 ± 0.1	6.4 ± 0.1
CCP	5.5 ± 0	4.8 ± 0.3	4.6 ± 0.2	6.0 ± 0.2	5.7 ± 0.2
CAY	5.2 ± 0.2	2.0 ± 0.3	3.4 ± 0.8	6.7 ± 0.2	6.1 ± 0.2
Mtail ⁻	≥6.5 ± 0	3.8 ± 0.2	3.0 ± 0.3	5.5 ± 0.2	4.5 ± 0.4
Tail ⁻	5.2 ± 0.2	2.8 ± 0.3	2.7 ± 0.3	7.0 ± 0.1	7.0 ± 0.3

^a Mice received either 2.0, 3.0, 4.0, 5.0, or 6.0 log₁₀ PFU of virus on day 0 in a 0.05-ml inoculum.

^b Transfectant virus MAY was not tested in mice.

^c Determined by the Karber method (15). Results are for six animals up to day 20.

^d Mean for six animals. The lowest detectable titer is 1.5 log₁₀ 50% tissue culture infective dose (TCID₅₀) per g.

no reversions were found in the introduced substitution sites of the other cysteine-altered viruses.

Genetic stability of the HA cysteine-altered transfectant viruses. We found previously in an examination of the genetic stability of the tail⁻ HA transfectant virus that revertants could be isolated readily (10), suggesting that influenza virus prefers possession of an HA cytoplasmic tail. To examine the genetic stabilities of Mtail⁻ (which contains three consecutive translational stop codons), CAC, CCA, CCP, CAY, and MAY, the viruses were passaged in eggs continuously for five generations, virion RNA was extracted, and the nucleotide sequence of the relevant portion of RNA segment 4 encompassing the introduced mutations was determined for plaques of each transfectant virus. In only one plaque of five for CAY transfectant virus was a change found: the introduced codon TAT, encoding tyrosine, reverted to TGT, which encodes cysteine.

Membrane fusion analysis of the HA cysteine-altered mutants. To investigate, in the context of infectious virions, whether mutations in the HA cytoplasmic tail cysteine residues, or deletion of the HA tail, affects the kinetics and extent of fusion, we used the R18 lipid-mixing assay, which measures mixing of the outer-leaflet membrane lipids (12). As shown in Fig. 4A, it was found that Mtail⁻ virus displayed an initial fusion rate and final extent of fusion similar to those of wt HA transfectant virus. Each of the other HA cysteine-altered viruses had kinetics (Fig. 4B) and extents of fusion (data not shown) similar to those of wt HA virus. The pH optima to initiate fusion in the R18 fluorescence dequenching lipid-mixing assay were identical for the wt HA and Mtail⁻ transfectant viruses (pH 5.5).

Recently, it has been shown that for complete fusion, i.e., the mixing of inner-leaflet membrane lipids with concomitant mixing of aqueous contents of donor and recipient cell, to occur, the HA transmembrane domain is required (12). To investigate whether the HA cytoplasmic domain might affect complete fusion, we used a content-mixing assay to compare the most severely altered transfectant virus (Mtail⁻ HA) with the wt HA transfectant virus. At 5 h p.i., virus-infected CV-1 cells were treated with TPCK-trypsin to activate HA and also treated with NA to remove sialic acid from the cell surface to prevent fusion between CV-1 cells. The cells were then overlaid with NBD-aurine-loaded human erythrocytes, fusion between the two cell types was initiated by incubation in low-pH medium (pH 5.0), and the cells were observed under a fluo-

TABLE 3. Infectivities of rescued viruses in MDCK cells^a

Virus	Titer (PFU/ml [10 ⁵])	HA/ml	PFU/HA (10 ³)	PFU/HA normalized to that of wt HA
wt HA	7.5	512	1.46	1.0
CAC	7.5	256	2.93	2.00
CCA	0.7	32	2.19	1.50
CCP	2.0	64	3.13	2.14
CAY	0.5	64	0.78	0.53
MAY	1.3	128	1.01	0.69
Tail ⁻	3.0	256	1.17	0.80
Mtail ⁻	1.5	128	1.17	0.80

^a MDCK cells were infected with viruses at a multiplicity of infection of 0.01 PFU per cell and incubated at 37°C for 3 days. The infectivity was determined by plaque assay in MDCK cells.

FIG. 4. Fusion kinetics of wt HA virus and HA cytoplasmic tail cysteine mutants. Purified transfectant virions were labeled with octadecyl rhodamine B (R18) and incubated with MDCK cells for 1 h at 4°C. Three milliliters of prewarmed PBS was added to a thermostatted cuvette in a spectrofluorimeter, and to the cuvette was added an aliquot of R18-labeled virion-MDCK complexes. Baseline data were recorded for 20 s, and then fusion was initiated by lowering the pH of the contents of the cuvette to 5.0 by addition of a predetermined amount of 0.25 M citric acid. (A) Kinetics of fluorescence dequenching (FDQ) of R18-labeled wt HA and Mtail⁻ HA viruses. (B) Rates of fluorescence dequenching of wt HA virus and the HA cytoplasmic tail cysteine mutants, calculated from the maximal slopes of curves analogous to those shown in panel A.

rescence photomicroscope. As shown in Fig. 5, on lowering of the pH to 5, the fluorescent NBD-aurine was transferred to the CV-1 cells. The rate of content mixing of the NBD-aurine cannot be measured by this method, but quantification of the fluorescent signal detected by the microscope luminometer indicated that the fluorescence intensities from the pH 5.0-treated cells were very similar for the two viruses. No content mixing was observed for cells expressing uncleaved HA₀ and treated at pH 5.0. Thus, these data indicate that the lack of an HA cytoplasmic tail, in the context of an infectious virus, does not affect the steps of membrane fusion activity examined.

DISCUSSION

The study reported here shows that influenza virus containing the Mtail⁻ HA mutation can be rescued. Thus, the conclusion that an HA cytoplasmic tail is not needed for incorporation of HA into virions or for the budding of particles at the plasma membrane (10) is strengthened. The Mtail⁻ HA mutant virus contains three consecutive translational stop codons, which makes suppression of termination or reversion to tail⁺ HA a remote possibility. Furthermore, in this study we also showed that HA cysteine residues 555, 562, and 565 per se are not important for incorporation of the normal amounts of polypeptides NA, NP, M₁, and M₂ into virions when the virus is grown in eggs, as the triple mutant MAY efficiently formed virions that contained normal amounts of these proteins. Cysteine residues 555, 562, and 565 in the wt HA virus can be modified by the addition of palmitate through a covalent thioether linkage, and thus the ability to rescue the Mtail⁻ HA and MAY viruses also indicates that palmitylation is not essential for virus assembly. In the electron microscope, all of the mutants described here had similar spherical morphologies (reference 10 and data not shown).

In addition to our findings that the conserved cytoplasmic tail of HA is not required for virus particle formation, it has

been found that the conserved cytoplasmic tail of NA is not required for virus formation (7, 11). As the formation of a patch of budding virus does not appear to be a stochastic process, it seems reasonable to assume that there are protein signals that specify protein-protein interactions. In this regard, the 54-residue cytoplasmic tail of the M₂ protein may be important for virus assembly, as well as for possible interactions between the hydrophobic domains of the matrix (M₁) protein and the TM domains of NA, HA, and M₂.

The infectivities of the cysteine-altered HA (H3 subtype) viruses and the tail⁻ HA viruses varied with the host for virus growth. Whereas all of the rescued viruses grown in MDCK cells had up to a 2-fold-lower PFU/HA ratio, the rescued viruses grown in embryonated eggs showed up to a 23-fold-lower PFU/HA ratio (e.g., CCP [Table 2]). The rescued Mtail⁻ HA virus (which lacks the addition of palmitate) had up to a fourfold-lower PFU/HA ratio when grown in eggs or MDCK cells, which suggests that palmitylation is not very important for infectious-virus formation. Furthermore, rescued virus CAC had a PFU/HA ratio in eggs and MDCK cells that was 1.1- to 2-fold higher than that of wt HA virus, and cysteine 562 in wt HA has the highest level of palmitylation (17). Thus, although we cannot exclude some role of palmitylation in contributing to infectivity in eggs or tissue culture, it seems likely that the 8- to 16-fold-lower PFU/HA ratio of rescued viruses CCA, CCP, CAY, and MAY compared with that of wt HA virus is because the amino acid substitution at cysteine 565 has a dominant negative effect. When the replication of the cysteine-altered HA viruses and the tail⁻ HA viruses was examined in mice, only virus CAC had properties similar to those of wt HA virus. The other viruses were attenuated for growth in the upper respiratory tract (3 to 4 log units lower than that of wt HA) but were less restricted in the lower respiratory tract (0.5 log unit [tail⁻ HA] to 2 log units [Mtail⁻ HA] lower than that of wt HA). Overall, the data suggest that rescued viruses

FIG. 5. Fusion activity measured by the content-mixing assay. wt and Mtail⁻ HA transfectant virus-infected CV-1 cells were treated with 0.2 mg of NA per ml and 10 μ g of TPCK-trypsin per ml for 10 min at 5 h p.i. and then incubated with NBD-taurine loaded erythrocytes. The pH was lowered to 5.0 for 1 min at 37°C or maintained at the neutral value, and after 5 min the cells were photographed on a Nikon inverted phase-contrast microscope.

(except CAC) have a host range restriction in the upper respiratory tract in the mouse.

Previously, we had observed that we could obtain a revertant virus of tail⁻ HA by reversion of the single translational stop codon to a codon encoding tryptophan (10). With the H1 HA subtype, Zurcher and colleagues (38) found that the mutant CYC (which in that system was growth impaired) reverted to CCC. Here we report a revertant of virus CAY in which tyrosine 565 has changed back to cysteine. In the latter two cases, a single nucleotide change in the tyrosine codon could have resulted in a codon encoding phenylalanine, histidine, asparagine, aspartic acid, or serine. Although in neither case were enough revertants examined to determine whether other residues could be tolerated at these positions, the natural selection approach does suggest that the conserved sequence of the HA cytoplasmic tail confers an advantage.

As the introduction of changes into the HA cytoplasmic tail did not cause profound differences in the polypeptide compositions of assembled virions or greatly affect virus infectivity, it seemed possible that the mutations could affect an aspect of fusion. Even though the HA cytoplasmic tail mutations do not affect the abilities of the rescued viruses to cause syncytium formation (an end step in fusion) (our unpublished observations) and as the rescued viruses replicate they have to be capable of fusing with endosomal membranes, the HA mutations could affect the kinetics of fusion. Recently, it has been found by using the R18 assay that HA containing a glycosyl phosphatidyl inositol anchor (and lacking a TM domain) causes only hemifusion, i.e., mixing of the outer lipid leaflet but

not the inner lipid leaflet (12). Thus, it seemed possible that alterations to the HA cytoplasmic tail could affect the kinetics of fusion when measured with the R18 assay. However, our experimental evidence indicates that none of the HA cytoplasmic tail mutants exhibited a major change in fusion kinetics.

Studies related to those described here have been performed with the H1 HA subtype (38). The three HA₂ C-terminal cysteine residues were replaced with various residues, and attempts to rescue transfectant viruses were made. It was found that rescued viruses could be obtained only for cysteine residues equivalent to H3 subtype HA residues 555 and 562. Rescued virus with changes in the cysteine residue equivalent to H3 HA residue 562 had reduced infectivity. No viruses containing changes in the cysteine residue equivalent to H3 HA residue 565 could be rescued, nor could double mutants with changes in cysteine residues equivalent to H3 HA residues 555 and 562 be isolated (38). Possible explanations for the differences in the experimental data obtained with H1 and H3 HA transfectant viruses include the following. (i) It is possible that amino acid changes in the cytoplasmic tail of the H1 subtype HA are tolerated less well in forming an infectious virion than the same changes in the H3 subtype HA. The reverse genetics transfectant-rescue system for influenza virus is currently not sufficiently efficient to make it possible to isolate viruses that are attenuated in virus growth by more than 2 to 3 log units. (ii) Since different specific changes (except CAC) were made in the H1 and H3 HA subtypes (reference 38 and the present study), it is possible that the specific changes made in each case are the determining factor for infectivity rather than the sub-

type of HA. In this regard, in limited experiments, we did not succeed in rescuing viruses with the changes CAA, AAC, ACA, and AAA. The maintaining of the conserved sequence of the HA cytoplasmic tail in naturally occurring viruses, the tendency for mutant viruses to revert back to cysteine residues, and the differences in replication in vitro, in ovo, and in vivo observed in the present study indicate that the HA cytoplasmic tail plays a very important role in the generation of virus that can replicate in multiple cell types. Moreover, an implication of these findings is that infection of the diverse cells throughout the respiratory tract is likely important for highly efficient transmission of viruses and thus that viruses that can multiply in multiple cell types will be naturally selected.

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