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Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape

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The cytoplasmic tails of the influenza virus glycoproteins hemagglutinin (HA) and neuraminidase (NA) are highly conserved in sequence for all virus subtypes and it is believed that assembly of this enveloped virus depends on interactions of these domains with cytoplasmic viral components. However, it is possible to rescue altered influenza viruses lacking either the HA or NA cytoplasmic tails. We have obtained an influenza virus that lacks both the cytoplasmic tail of HA and NA. Particle production is reduced ~10-fold but these particles, although having a fairly normal protein composition, are greatly elongated and of extended irregular shape. We propose a model in which the interactions of the cytoplasmic tails of HA and NA with an internal viral component are so important for spherical virion shape that there is dual redundancy in the interactions.

Keywords: cytoplasmic tails/hemagglutinin/influenza virus/neuraminidase

Introduction

Many enveloped viruses mature by budding at the plasma membrane of infected cells. In the budding process, the viral core complex is enveloped by the cellular plasma membrane which contains viral spike glycoproteins. For many years it has been viewed that interactions between the cytoplasmic tails of the viral glycoproteins and the internal components of the virion trigger the formation of the budding particle (reviewed in Compans and Choppin, 1975; Simons and Garoff, 1980; Dubois-Dalcq *et al.*, 1984). For the alphavirus, Semliki Forest virus, an interaction between the cytoplasmic tail of the E2 spike glycoprotein and the core has been shown to be essential for budding (Suomalainen *et al.*, 1992; Zhao *et al.*, 1994). In contrast, for the retrovirus Rous sarcoma virus (RSV), infectious virions are released from cells when the glycoprotein (gp37) lacks its cytoplasmic domain (Perez *et al.*, 1987).

Studies on the requirements for glycoprotein cytoplasmic tails in the budding of enveloped viruses that contain helical ribonucleoprotein capsids (RNPs) had yielded data that was open to several interpretations. However, with the development of reverse genetic systems for influenza virus, rhabdoviruses and paramyxoviruses (Luytjes *et al.*, 1989; Enami *et al.*, 1990; Schnell *et al.*, 1994; Collins *et al.*, 1995; Garcin *et al.*, 1995; Lawson *et al.*, 1995; Radecke *et al.*, 1995; Whelan *et al.*, 1995), it has been possible to begin to examine the budding process and obtain more unambiguous data. For rhabdoviruses, from studies using vesicular stomatitis virus, it had been thought that efficient inclusion of the spike G protein in virions depended on interactions with the viral matrix (M) protein, the RNP or both (Whitt *et al.*, 1989; Owens and Rose, 1993). However, recent studies with another family member, rabies virus, using mutants in which the G protein cytoplasmic tail or the entire G protein was deleted from the genome, indicate that particles of normal morphology can be produced from cells, although ~6- (G tail–) or 30-fold (G minus) less efficiently than wild-type (wt) virus (Mebatsion *et al.*, 1996).

The influenza A virus envelope contains two major glycoproteins, hemagglutinin (HA) and neuraminidase (NA) and, in much lower abundance, the M_2 ion channel protein. Contained within the influenza virus envelope are the viral matrix (M_1) protein and the eight segments of the viral RNPs (reviewed in Lamb and Krug, 1996). The HA cytoplasmic tail contains 10 or 11 C-terminal residues, under the assumption that the charged residue adjacent to the hydrophobic transmembrane (TM) domain delineates the boundary of the domain, and these residues are highly conserved among the 14 HA subtypes (Nobusawa *et al.*, 1991) (Figure 1). The NA cytoplasmic tail contains six N-terminal residues of identical sequence (MNPNQK) for all nine known NA subtypes, with the exception of two isolates (Blok and Air, 1982; reviewed in Colman, 1989). Given the mutation rate of the RNA genome of influenza virus, the sequence conservation of the HA and NA cytoplasmic tails suggests that they must be maintained for an important function.

By using reverse genetics, it was very interesting to find that rescued (transfectant) influenza viruses could be obtained that lacked either the cytoplasmic tail of HA (HAt–/NA) or the cytoplasmic tail of NA (NAt–) (nomenclature described below) (see Figure 1). The HAt–/NA virus had normal incorporation of HA and other virion polypeptides and exhibited the normal spherical morphology of egg-grown virus but had a slightly lower budding efficiency and was slightly less infectious than wt virus (Jin *et al.*, 1994, 1996). The NAt– virus in the same genetic background was isolated by two different groups. The NAt– virions isolated by García-Sastre and Palese (1995) had properties similar to wt virions except for containing a decreased amount (65%) of NAt– protein and having a 1 log lower infectivity in tissue culture, but their morphology was not examined. In contrast the NAt– virus isolated by Mitnaul and co-workers (1996) contained only 14% of the normal amount of NA protein, had a

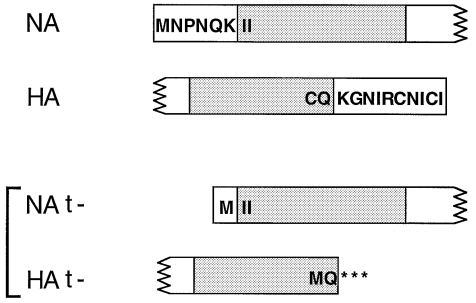

Fig. 1. Schematic diagram of the cytoplasmic tails of the influenza virus NA and HA. The transmembrane domains of the NA and HA integral membrane proteins are presumed to be a hydrophobic region bound by charged residues and, using this demarcation of domains, the sequences of the cytoplasmic tails are indicated. NA is a type II integral membrane with a single N-terminal hydrophobic domain that acts as both a signal sequence and as an anchor domain. The sequence of the NA N-terminal six residue cytoplasmic tail is indicated. HA is a type I integral membrane protein with a C-terminal anchorage domain and a 10 residue cytoplasmic tail. The mutant containing a NA with a deleted cytoplasmic tail (NAt–) has the N-terminal initiation methionine residue fused to the first isoleucine residue of the transmembrane domain as was described previously (García-Sastre and Palese, 1995). The mutant containing an HA with a deleted cytoplasmic tail (HAt–/NA) has three consecutive translational stop codons placed after the glutamine in the transmembrane domain; in addition, the penultimate residue of the transmembrane domain, Cys555, was substituted with methionine as was described previously (Jin *et al.*, 1996). A genetic reassortant containing the altered RNA segments of the rescued (transfectant) influenza viruses containing the NAt– and HAt– mutations was isolated to yield a virus designated HAt–/NAt–.

2 log lower infectivity, produced very small plaques and virions were observed to aggregate at the cell surface and had a somewhat altered morphology (exhibited a tendency to form more filamentous particles than largely spherical particles). The reason for the difference in these data is not known, but it is possible that either NAt– virus contained mutations in other RNA segments. The ability to recover the HAt–/NA virus and NAt– viruses and their properties suggested that the role of each cytoplasmic tail, although probably important for virus replication, was not absolutely essential. Thus, it became important to determine if an influenza virus could be obtained that contained deletions of both the HA and NA cytoplasmic tails (HAt–/NAt–).

We describe here the generation of a HAt–/NAt– double mutant virus and we show that the virions released from the infected cell are of greatly altered morphology: vastly extended lengths and irregular diameters. We propose a model in which, for the normal budding of influenza virus, the requirements for glycoprotein cytoplasmic tail interactions with an internal virion component (most likely the M_1 protein) are so critical that these cytoplasmic tail interactions are dually redundant.

Results

Isolation of an influenza virus reassortant lacking cytoplasmic tails to HA and NA

To attempt to obtain an influenza virus that lacked the cytoplasmic tails of HA and NA, we considered the chances of success would be greatest if we used the least technologically sophisticated approach, i.e. isolation of a reassortant virus from a mixed infection. First, the subtype H3 HA lacking its cytoplasmic tail (HAt–) and the wt H3 HA (HA), which were in the genetic background of strain A/WSN/33 (H1N1) (Jin *et al.*, 1994, 1996), were transferred to the genetic background of strain A/Japan/305/57 (H2N2). These reassortants were designated HA/Japan and HAt–/Japan (H3N2), and they were used as intermediates in a further mixed infection with NAt–, a rescued virus (WSN) that lacks the NA cytoplasmic tail (García-Sastre and Palese, 1995). UV-irradiated HA/Japan and HAt–/Japan were inoculated into the allantoic cavity of embryonated eggs along with NAt– virus and polyclonal sera specific for N2 subtype NA and H1 subtype HA. Reassortant viruses were purified in Madin–Darby canine kidney (MDCK) cells using the endpoint dilution method and then plaqued in MDCK cells. Although neither the HAt–/NA nor the NAt– viruses used in this study showed a reduction in the size of plaques formed, the HAt–/NAt– virus formed very small plaques on MDCK cells.

Multiple examples of the reassortant viruses HA/NA, HA/NAt– and HAt–/NAt– were isolated: HA indicates a virus with a H3 subtype HA, NA indicates a virus with a N1 subtype NA and t– indicates deletion of the cytoplasmic tail of the protein. To verify that these reassortant viruses contained the N1 subtype NA genes, cDNA of the NA gene was PCR amplified using N1 subtype- and N2 subtype-specific oligonucleotides. As shown in Figure 2A, HA/NAt– and HAt–/NAt– contained the N1 NA gene. The nucleotide sequence of the appropriate region of the cDNA confirmed that HAt–/NAt– contained a deletion in the region encoding the NA cytoplasmic tail (Figure 2B) and the NA gene did not contain other changes in the region encoding the transmembrane domain, as had been found for some isolates of NAt– (Mitnaul *et al.*, 1996). To examine the synthesis and antigenic nature of the NA protein of the HA/NAt– and HAt–/NAt– viruses, NA protein was immunoprecipitated from metabolically labeled virus-infected MDCK cells using N1-specific or N2-specific sera. As shown in Figure 2C, the NA protein of viruses WSN, NAt–, HA/NAt– and HAt–/NAt– could be immunoprecipitated by the N1-specific sera but not by the N2-specific sera. The electrophoretic mobility of the NAt– protein was slightly faster than that of the wt NA of WSN virus, but it is known that the deletion of the six residue NA cytoplasmic tail causes only small changes in gel mobility (García-Sastre and Palese, 1995). The N2 NA of A/Japan/305/57 (JAP) showed its characteristic electrophoretic mobility and antigenic reactivity. To examine the synthesis of the HA species of the HA/NAt– and HAt–/NAt– viruses, metabolically labeled virus-infected MDCK cell lysates were treated with TPCK-treated trypsin to cleave HA to HA_1 and HA_2, and HA species were immunoprecipitated. As shown in Figure 2C, the HA_2 subunit of HAt–/NAt– virus, but not HA/NAt– virus, showed a characteristic faster gel mobility than wt HA_2, as was observed previously (Jin *et al.*, 1994, 1996). Genotyping of HA/NAt– and HAt–/NAt– viruses for RNA segment 5 encoding the nucleocapsid protein (NP), RNA segment 7 encoding the M_1 and M_2 proteins, and RNA segment 8 encoding the NS_1 and NS_2 proteins was performed. Examination of the differences in gel mobilities between the polypeptides of the parental types (HA/Japan or HAt–/Japan and WSN NAt–) indicated that for HA/NAt– and HAt–/NAt–, RNA segments 5, 7 and 8 were of

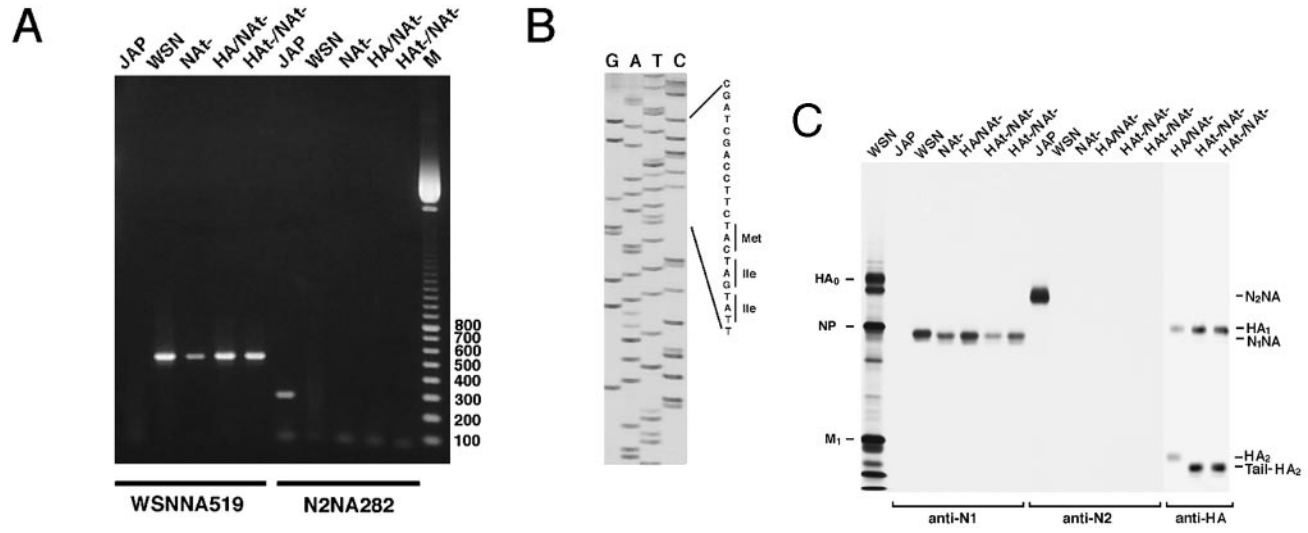

Fig. 2. Biochemical characterization of an influenza virus lacking the HA and NA cytoplasmic tails (HAt–/NAt–). (**A**) Identification of the NA segment of HAt–/NAt– and HA/NAt– reassortants by RT–PCR. Viral RNA was extracted from allantoic fluid of egg-grown viruses. cDNA was synthesized using an N1 NA-specific oligonucleotide (N1NA5′) and was PCR amplified with N1NA5′ and N1NA519 (left panel) or cDNA was synthesized with an N2 NA-specific oligonucleotide (N2NA5′) and PCR amplified with N2NA5′ and N2NA282 (right panel). The PCR products were analyzed on a 1% agarose gel. JAP = influenza virus A/Japan/305/57; WSN = influenza virus A/WSN/33; NAt– = influenza virus transfectant containing WSN NA (N1 subtype) with a deletion of its cytoplasmic tail in a WSN genetic background. HA/NAt– = influenza virus containing the A/Udorn/72 HA (H3 subtype) and NAt–; HAt–/NAt– = influenza virus containing the H3 HA with a cytoplasmic tail deletion (HAt–) and NAt–. (**B**) Sequence analysis of the NA RNA segment of the HAt–/NAt– virus. The nucleotide sequence of the PCR product of HAt–/NAt– was determined using a primer N1NA134–152. The linker sequence which was inserted between the non-coding region of the gene and the ATG codon and which replaces the nucleotides deleted from the gene is indicated, and the initiation codon (ATG) and the two downstream isoleucine codons in the transmembrane region are also indicated. (**C**) Antibody reactivity of HA and NA synthesized by the reassortant viruses HA/NAt– and HAt–/NAt–. MDCK cells were infected with the viruses indicated and at 4.5 h post-infection were metabolically labeled with Tran[^{35}S]-label for 2 h. Aliquots of cell lysate were immunoprecipitated with antisera specific for N1 NA, N2 NA or H3 HA. Note the faster migration of the NAt– species compared with N1 NA. No N2 NA was detected in the reassortants HA/NAt– and HAt–/NAt–. To analyze the mobility of the HA_2 subunit of HA, infected cell lysates were incubated with TPCK-trypsin to cleave HA_0 to HA_1 and HA_2, and the proteins were immunoprecipitated using an H3 HA-specific antibody. Note that HA_2 of virus HAt–/NAt– migrates faster than HA_2 of virus HA/NAt–. Two independent isolates of HAt–/NAt– are shown.

A/WSN/33 origin (data not shown). The origin of the viral transcription complex-associated proteins, PB1, PB2 and PA, was not determined, but it seems extremely unlikely that these stoichiometrically minor components of the nucleocapsid core would affect the assembly process of the virus.

Polypeptide and RNA composition of the HAt–/NAt– virus

To analyze the biochemical properties of HA/NAt– and HAt–/NAt– viruses and to compare them with HA/NA virus, the viruses were grown in eggs and purified on sucrose density gradients. On rate zonal centrifugation, all the virions sedimented as discrete bands but the HAt–/NAt– band was broader than that of HA/NA virus. On equilibrium centrifugation, the virions had a density of ~1.18–1.19 g/cm^2 and it was not possible to distinguish HA/NA virus from HAt–/NAt– virus unequivocally by density. The polypeptide composition of the virions was analyzed by SDS–PAGE and the polypeptides were visualized by staining with Coomassie brilliant blue (Figure 3A). HA_1, NP and NA migrate closely on gels, and thus to obtain a better characterization of these species, virions were digested with peptide *N*-glycosidase F to remove *N*-linked carbohydrate chains and to alter the mobility of HA and NA species (Figure 3B). To confirm the mobilities of the NA species, immunoblotting of a parallel gel was performed using N1 NA-specific sera (Figure 3B insert). As would be expected, the HA_2 subunits of virions expressing HAt– migrated significantly faster that wt HA, and NAt– migrated faster than wt NA. Furthermore, the relative abundances of the viral polypeptides HA_1, HA_2, NA, NP and M_1 were not vastly altered in the viruses by the absence of an HA and/or NA cytoplasmic tail (compare lanes HA/NA with HAt–/NA, HAt–/NAt– and HA/NAt–). None the less, the amount of HA protein in HAt–/NAt– virions as normalized to NP was increased ~5-fold (as determined by densitometry) when normalized to the amounts of these polypeptides in HA/NA virions (Table I). However, it is emphasized that NP is used here only as a reference point as we do not know if NP is involved in the process of particle formation and if its amount changes between HA/NA and HAt–/NAt– viruses.

To determine the relative amount of the M_2 ion channel protein incorporated into the HA and NA cytoplasmic tail-altered virions, immunoblotting of virion polypeptides was performed using M_2-specific antibodies and, as a control for protein loading, H3 HA-specific antibody. As shown in Figure 3 (lower panel), M_2 protein was readily detected in all the virion preparations, mostly as a doublet of intact M_2 and proteolytically clipped M_2 protein, as has been observed previously in preparations of WSN virions (Zebedee, 1988; Panayotov and Schlesinger, 1992). Interestingly, all altered virions containing NAt– incorporated 3- to 6-fold more M_2 protein than virions containing wt NA.

It is a possibility that the NP/HA/M_2 ratio change observed between HA/NA and HAt–/NAt– viruses is due

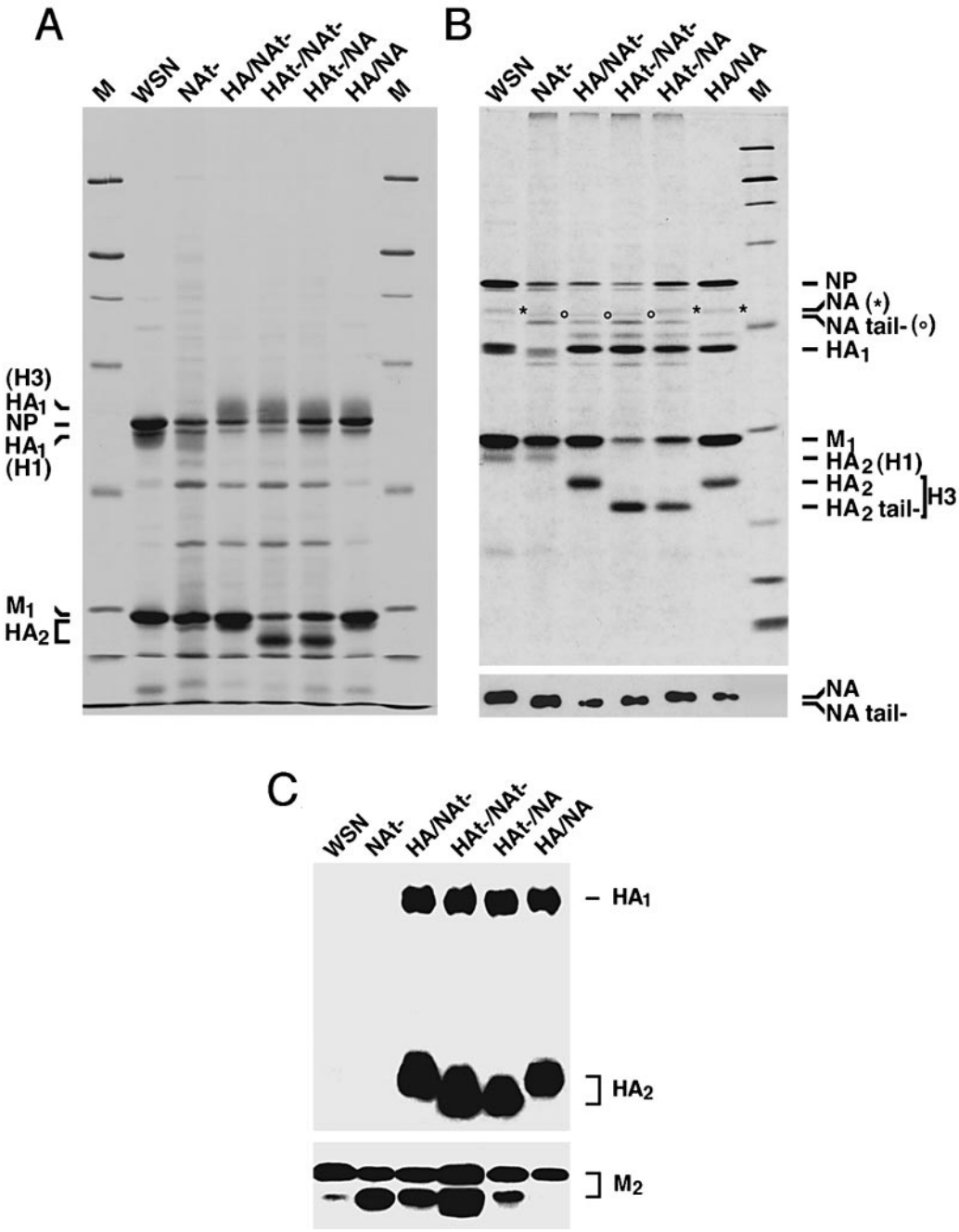

Fig. 3. Protein composition of influenza viruses with altered HA and NA cytoplasmic tails. (**A**) Viruses were grown in embryonated chicken eggs, the allantoic fluid harvested and virions purified by centrifugation through 20–60% sucrose gradients. Purified virions (~15 μg protein) were subjected to SDS–PAGE on a 10% gel and the polypeptides were stained with Coomassie brilliant blue. Lanes WSN, NAt–, HA/NAt– and HAt–/NAt– are as described in the legend to Figure 2. HAt–/NA = transfectant influenza virus containing H3 HA with a cytoplasmic tail deletion and a wt N1 NA. HA/NA = transfectant influenza virus containing wt H3 HA and wt N1 NA. M = marker polypeptides. (**B**) Purified virions (~15 μg protein) were digested with peptide *N*-glycosidase F to remove *N*-linked carbohydrate chains in an attempt to separate NA from HA_1 and NP more readily. Polypeptides were analyzed by SDS–PAGE on a 15% gel and stained with Coomassie brilliant blue: (∗) indicates deglycosylated NA and (o) indicates deglycosylated NAt–. Lower panel: immunoblotting of a parallel gel to that shown above using a N1 NA-specific serum to confirm the electrophoretic mobility of the deglycosylated NA species. (**C**) Immunoblotting of HA: aliquots of the viruses (0.5 μg) were subjected to SDS–PAGE on 17.5% gels containing 4 M urea and polypeptides transferred to membranes and immunoblotted using a serum specific for H3 HA. Lower middle, lower panel: immunoblotting for M_2 protein. Aliquots of the viruses (1 μg) were subjected to SDS–PAGE on 17.5% gels containing 4 M urea and polypeptides transferred to membranes and immunoblotted using mAb 14C2 specific for M_2 protein.

not to changes in the cytoplasmic tails, but to changes in the ratios of proteins synthesized in virus-infected cells. For example, an adventitious mutation could have arisen during the production of these mutants, causing differential expression of RNA segments. Therefore, the levels of viral polypeptides synthesized in virus-infected cells were examined for all the mutants and no changes were observed (data not shown).

To investigate the neuraminidase activity of NA and NAt– incorporated into virions, the enzymatic activity of purified virions was tested by incubating with the disaccharide neuraminlactose as substrate and measuring

Table I. Amounts of polypeptides in rescued virions normalized to that of NP

Polypeptide	Relative amount in[a]			
	HA/NA	HAt–/NA	HA/NAt–	HAt–/NAt–
HA	2.19	2.9	7.28	10.04
NA	0.14	0.29	0.12	0.25
M_1	2.09	1.55	6.48	2.40

[a]The Coomassie brilliant blue-stained gel shown in Figure 3 top right was scanned with a densitomer and the relative staining intensity of each polypeptide was normalized to that of NP (set at 1.00) for each transfectant virus. Only viruses containing the H3 HA are reported, as the H1 HA binds a different amount of Coomassie brilliant blue making a cross-comparison not useful.

the released *N*-acetyl neuraminic acid (NANA). NA specific activity was calculated as μg of NANA released per mg of total viral protein. As shown in Table II, all the virions had similar neuraminidase activities.

To determine the RNA segment composition of HA/NA and HAt–/NAt–, RNA was extracted from purified virions and analyzed on 3.5% polyacrylamide gels containing 8 M urea, and the RNA segments were visualized by staining with ethidium bromide. The relative ratio of the eight RNA segments between HA/NA and HAt–/NAt– virions was found to be indistinguishable (data not shown; see Jin *et al.*, 1994). However, the RNA–protein ratio of HA/NA was 1.0 μg of RNA to 60 μg of protein, which is consistent with the estimated composition of influenza viruses (0.8–1% RNA, ~70% protein, ~20% lipid and 5–8% carbohydrate; reviewed in Compans and Choppin, 1975), whereas for HAt–/NAt– the ratio was found to be 0.5 μg of RNA to 60 μg of protein. The lower amount of RNA for HAt–/NAt– is consistent with the lower ratio of NP as compared with HA.

Infectivity of HAt– and NAt– cytoplasmic tail deletion viruses

The infectivity of the HAt– and NAt– cytoplasmic tail deletion viruses grown in embryonated eggs or in MDCK cells was determined by the tissue culture infectious dose endpoint dilution ($TCID_{50}$) method and by plaque assay. As discussed above, HAt–/NAt– virus produce small (1 mm diameter in 5 days) plaques in comparison with HA/NA virus (3 mm diameter in 3 days). Egg-grown HAt–/NAt– virus had the lowest infectivity, as measured by $TCID_{50}$ and plaque-forming units (p.f.u.), as compared with the other HAt– and NAt– cytoplasmic tail deletion viruses (Table II). In comparison with the control HA/NA virus, the infectivity (p.f.u.) of the egg-grown HAt–/NAt– was reduced 13-fold and the quantity of particles produced, as measured by hemagglutination assay, was reduced 10-fold. MDCK cell-grown HAt–/NAt– showed an even greater reduction in infectivity ($TCID_{50}$ and p.f.u.) than the control HA/NA virus (Table III). The greater infectivity of HAt–/NAt– in eggs than in MDCK cells (~170-fold) could be interpreted as a host-range restriction of the HAt–/NAt– virus, but this does not appear so likely if it is taken into consideration that the HA/NA control virus exhibited a 26-fold lower infectivity in MDCK cells than in eggs. The infectivity of egg-grown virus particles across the peak fractions of virus obtained from a velocity sucrose gardient indicated that all fractions contained virions of similar infectivity.

Morphology of HAt– and NAt– cytoplasmic tail deletion viruses

As the protein and RNA composition of HAt–/NAt– virions was very similar to that of HA/NA virions yet the amount of virus released from cells and its infectivity was reduced as compared with control virus, it was of interest to examine the morphology of negatively stained virions in the electron microscope. Examples of electron micrographs for HAt–/NA, HA/NAt– and HAt–/NAt– are shown in Figure 4. The sizes of the virions were quantified by measuring their length in units of the diameter (average 75 nm) of spherical particles (first four columns of the histogram in Figure 5). Virions containing irregular bulges were recorded as those with extended diameters (last column, which is independent of the other columns, in Figure 5). HAt–/NA virions showed a population of virions with a predominantly spherical shape (60–90 nm), very similar in shape to HA/NA virions, as had been observed previously (Jin *et al.*, 1994). Thus, these data indicate that the loss of the HA cytoplasmic tail alone does not affect virion morphology greatly beyond the natural diversity of length of virions. HA/NAt– virions showed a population of particles with a shift towards virions of increased length (Figures 4 and 5), and such a trend had been recognized previously with NAt– virion particles described as filamentous (Mitnaul *et al.*, 1996). One of the more extreme examples of an elongated NAt– virion is shown in Figure 4 for comparison. For HA/NAt–, 14% of the population had a length over five times the average diameter and 17% of the population had irregular shapes. In contrast to the almost normal morphology of HAt–/NA virions, HAt–/NAt– virions exhibited a population of molecules with a dramatic shift towards particles of greatly increased length and irregular shapes (Figure 4) [41% of the population had a length over five times the average diameter and 51% of the population had irregular shapes (Figure 5)]. It should be noted that in Figure 4 the virion at the bottom left is at the same magnification as HAt–/NA, but for the other examples of HAt–/NAt– the magnification is lower. Although it is more difficult to find an adequate means of quantification of the irregular shaped viruses, the HAt–/NAt– virus exhibited very different irregular shapes (mostly larger diameters) with a much greater frequency than any of the other viruses: even the HAt–/NAt– virions with a length one to two normal diameters had irregular bulges. Thus, HAt–/NAt– virions could be distinguished from HA/NAt– or NAt– virions in blind experiments.

We also made attempts to examine virions budding from the surface of virus-infected cells by examining thin sections in the electron microscope. However, we were not able to find examples of the cell surface containing longitudinal sections of the irregular shaped HAt–/NAt– viruses due to the very high frequency of making tangential sections through particles: cross-sections of the HAt–/NAt– virions could be observed (data not shown). Furthermore, this problem was exacerbated by the lower amount of virus released from HAt–/NAt– virus-infected cells.

All the virions exhibited a layer of spikes projecting radially outward all over their surfaces (Figure 4). To examine further the distribution of HA, NA and M_2 in the

Table II. Infectivity, hemagglutination activity and neuraminidase activity of viruses grown in embryonated eggs

Virus	$Log_{10}TCID_{50}$/ml[a]	p.f.u./ml ($\times 10^6$)	HA/ml	HA/mg viral protein	NA activity[b] (μg NANA/μg protein)
HA/NA	6.57	40	1024	33 000	4.24
HAt–/NA	6.50	30	512	33 000	4.19
HA/NAt–	6.19	15	256	49 000	4.04
HAt–/NAt–	5.25	3	128	49 000	3.89

[a]$TCID_{50}$ = tissue culture infectious dose endpoint determined by method of Reed and Muench (1938). $Log_{10}TCID_{50}$/mg of viral protein was also determined for HAt–/NA (8.81), HA/NAt– (8.61) and HAt–/NAt– (8.53).
[b]NANA = μg *N*-acetyl neuraminic acid per μg viral protein released in a neuraminidase assay.

Table III. Infectivity of viruses grown in MDCK cells[a]

Virus	$Log_{10}TCID_{50}$	p.f.u./ml ($\times 10^4$)
HA/NA	6.20	150
HA/NAt–	5.48	35
HAt–/NA	4.42	50
HAt–/NAt–	3.62	1.75

[a]MDCK cells were infected with viruses at a m.o.i. of 0.01–0.05 p.f.u./cell and the virus infectivity was analyzed by tissue culture infectious dose (TCID) [with the 50% endpoint determined by the method of Reed and Muench (1938)] or plaque titration on MDCK cells.

HA/NA and HAt–/NAt– particles, virions purified on rate zonal and equilibrium sucrose density gradients were absorbed onto grids and stained with either HA-, NA- or M_2-specific antibodies. Following reaction with an appropriate secondary antibody coupled to 10 nm gold particles, the virus was negatively stained with phosphotungstic acid. As shown in Figure 6, the distribution of HA, NA and M_2 over the HAt–/NAt– virions appeared to be spread evenly and the pattern was not dissimilar to that observed for the control HA/NA virus, except for the larger size of the HAt–/NAt– particles. The spikes in Figure 6 appear to radiate further from the virions than in Figure 4, but this is due to the addition of both primary and secondary antibodies. Thus, all these data taken together indicate that the absence of both the HA and NA cytoplasmic tails leads to a loss of normal viral budding control resulting in the low level release of virions with increased length and extended irregular shapes.

Discussion

The assembly of influenza virus at the plasma membrane is thought to involve three major components, the RNP segments, the matrix protein (which associates with membranes and also the RNPs) and the cell surface-expressed integral membrane proteins HA, NA and M_2. Budding has been considered to be driven by interactions of the cytoplasmic tails of the integral membrane proteins with the viral internal components. The cytoplasmic tails of HA and NA are highly conserved in sequence in all subtypes of influenza virus, and when it became known that neither the HA nor the NA cytoplasmic tail was required for intracellular transport, or cell surface expression of HA or NA (Doyle *et al.*, 1985, 1986; Jones *et al.*, 1985; Simpson and Lamb, 1992; Jin *et al.*, 1994), the hypothesized role of the HA and NA cytoplasmic tails in budding was strengthened. None the less, by using reverse genetic systems, it was possible to rescue influenza viruses containing a deletion of the HA cytoplasmic tail (Jin *et al.*, 1994, 1996) or a deletion of the NA cytoplasmic tail (García-Sastre and Palese, 1995; Mitnaul *et al.*, 1996). However, revertant viruses were obtained for the rescued viruses that contained either an HA with a substitution of a single stop codon at the presumed boundary of the transmembrane and cytoplasmic domains [HA tail– virus (Jin *et al.*, 1994)] or an HA with a cytoplasmic tail substitution of Cys565 for tyrosine (Jin *et al.*, 1996), suggesting that possession of a cytoplasmic tail on HA does confer an advantage. Furthermore, HA molecules containing foreign cytoplasmic tails failed to be incorporated into virions that contained normal HA (Naim and Roth, 1993), suggesting a role for the HA cytoplasmic tail sequences in virus assembly. Thus, it was considered important to attempt to isolate a virus lacking the cytoplasmic tails to both HA and NA (HAt–/NAt–) as properties of such a virus might shed considerable light on the processes involved in the assembly of virions.

The finding that a reassortant virus could be isolated that lacked cytoplasmic tails to HA and NA demonstrates that the presence of these cytoplasmic tails is not absolutely required for budding. However, using erythrocyte hemagglutination as a titer of physical virus particles, the efficiency of virus production of HAt–/NAt– in eggs was reduced to 10% of control virus and the frequency of budding particles observed in the electron microscope was greatly reduced (data not shown). None the less, the polypeptide composition of egg-grown HAt–/NAt– was not grossly dissimilar to HA/NA virus but the ratio of HA relative to NP was ~5-fold higher than in the control virus. Furthermore, the RNA segment distribution in HAt–/NAt– appeared normal although the RNA:protein ratio was reduced ~2-fold as compared with control virus, which is consistent in the overall population of incorporation of the RNPs into the virions. When the infectivity of the egg-grown HAt–/NAt– virus was determined, it was found that this virus formed very small plaques on MDCK cells and had a 1 log lower infectivity (p.f.u.) than the control HA/NA virus. Thus, the lower infectivity and budding efficiency are due to the absence of the HA and NA cytoplasmic tails. However, the morphology of the released virions provided the greatest observed difference with HA/NA virus. HA/NA and HAt–/NA viruses formed mainly spherical particles, whereas HA/NAt– virus showed less regularity with an increased number of elongated and distended structures. However, the morphology of the double mutant HAt–/NAt– virions was profoundly altered, with a much greater frequency of elongated and distended

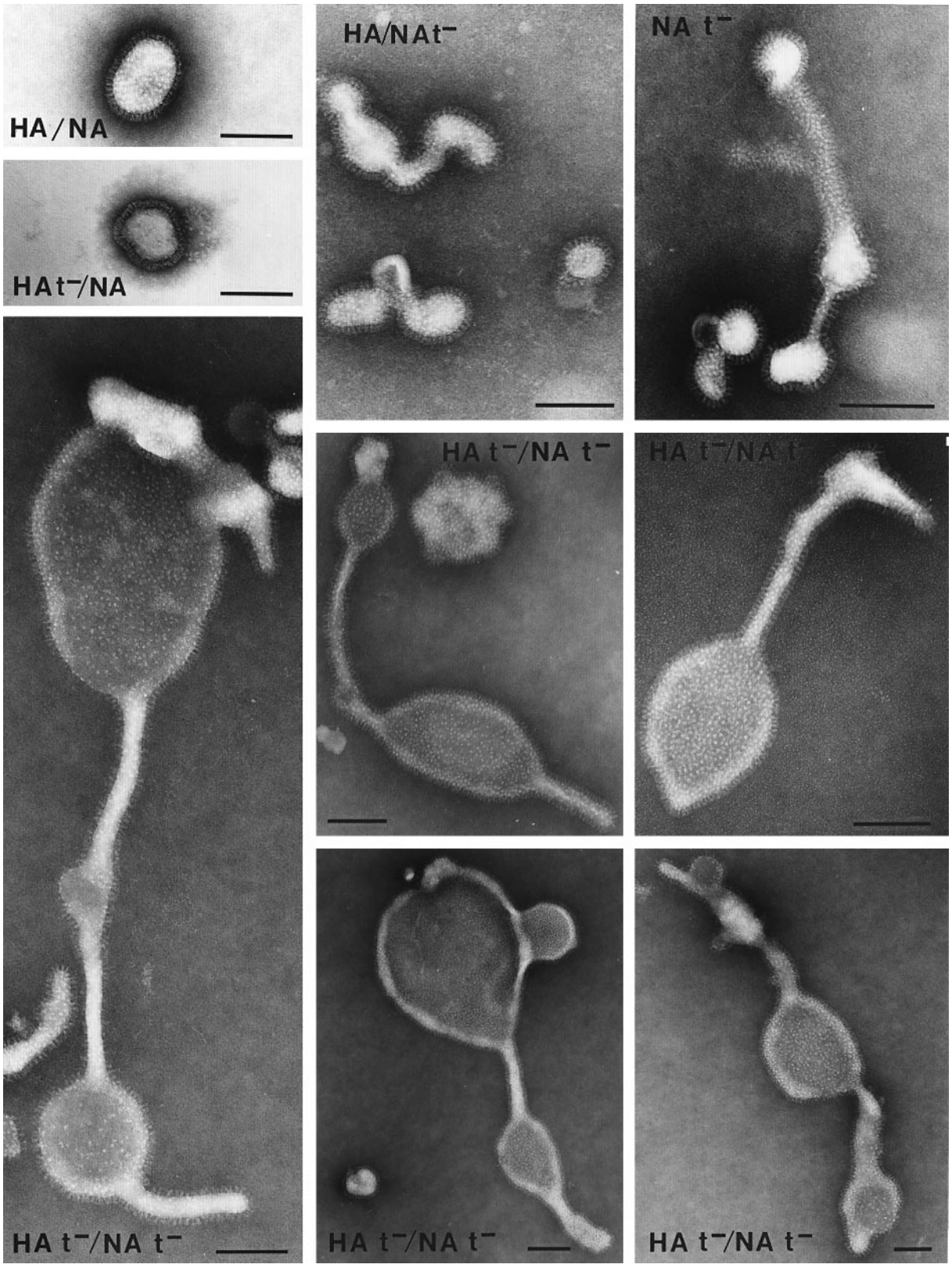

Fig. 4. Morphology of influenza viruses with altered HA and NA cytoplasmic tails. Virions were grown in embryonated eggs, purified on sucrose density gradients and absorbed onto Ni grids. Virions were negatively stained with phosphotungstic acid and examined by electron microscopy. Virus designations are as indicated in the legend to Figure 2. Bar = 80 nm.

structures. The morphology of these particles is somewhat reminiscent of those produced after treating influenza virus-infected cells with the lipid-intercalating compound, vitamin A alcohol (Blough, 1963), but for HAt–/NAt– virus the frequency of obtaining the elongated and distended structures was much greater.

Typically, influenza viruses adapted to high yield in tissue culture or eggs exhibit spherical morphology

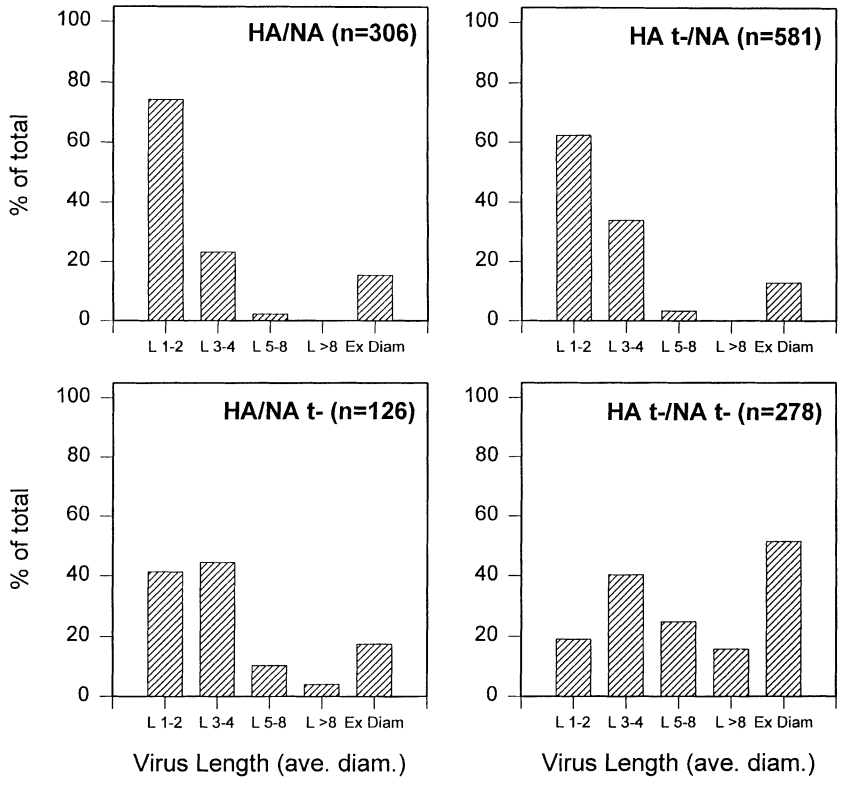

Fig. 5. Distribution of virus length and extended diameters as observed in the electron microscope of influenza viruses with altered HA and NA cytoplasmic tails. Random fields of negatively stained viruses were photographed and the length of the virions was measured in units of their diameter and recorded as 1–2, 3–4, 5–8, >8 times the average diameter. The percentage of virions containing irregular (extended) diameters was recorded as a separate column in the histogram. The number (*n*) of particles counted is given in each panel.

although all influenza viruses are pleomorphic. However, virus freshly isolated from infected humans or animals are often filamentous, with fairly uniform diameter particles (80 nm) and greatly elongated lengths (1–2 mm) (Burnet and Lind, 1957; Choppin *et al.*, 1960, 1961; Kilbourne and Murphy, 1960), but usually the morphology of these natural isolates is not irregular like the HAt–/NAt– virus. It has been considered that filamentous forms of viruses newly derived from man may represent inefficient replication during early passage in a new host, e.g. embryonated eggs (Kilbourne, 1987). Formation of filamentous influenza viruses is a genetic trait and is linked to the M_1 protein (Smirnov *et al.*, 1991). However, in our case, the possibility that the M_1 protein of HAt–/NAt– virus contained amino acid changes from the control HA/NA virus was eliminated when the sequences of RNA segment 7 encoding the M_1 protein of the two viruses were obtained and were found to be identical. None the less, if for the production of high yield spherical particles the cytoplasmic tails of HA and NA have to make efficient interactions with the M_1 protein, it can readily be envisaged that the mechanism for formation of the irregular shaped viruses is related to the observed conversion of low yielding filamentous virus to high yielding spherical particles: for filamentous virions, the interaction of spike protein cytoplasmic tails with M_1 protein may be suboptimal but, with time, changes in the M_1 protein sequence optimize the interactions and lead to the formation of spherical particles.

The visible spike density and distribution of immunogold labeling for HA and NA in the HAt–/NAt– virus was not obviously altered from HA/NA control virus and, from biochemical examination, the ratio of the amount of HA to NP was ~5-fold higher in HAt–/NAt– virus than in HA/NA virus. Thus, the specificity of incorporation of HA and NA spikes into particles was not hampered, unlike the case with another negative strand RNA virus, rabies virus, where it was found that a rescued virus containing a G protein with a cytoplasmic tail deletion caused a markedly lower quantity of spikes in virions (Mebatsion *et al.*, 1996). The influenza virus M_2 protein, which is normally greatly under-represented in virions in comparison with its surface expression level in infected cells (Zebedee and Lamb, 1988), was incorporated better into all viruses containing the NAt– change than into control virus. This may reflect a change in the postulated exclusion mechanism that in spherical particles keeps the level of M_2 protein at 5–15 tetramers per virion (Zebedee and Lamb, 1988). In this regard, it will be of great interest to determine if host cell membrane proteins are excluded from the HAt–/NAt– to the same extent as is believed to occur with wt virus (reviewed in Compans and Choppin, 1975). The influenza virus M_2 protein cytoplasmic tail is relatively long for a viral protein containing 54 residues (Lamb *et al.*, 1985), and an interaction of the M_2 protein with the M_1 protein has been suggested based on the observations that mutations in the M_2 cytoplasmic tail or the M_1 protein prevent the growth restriction caused by a monoclonal antibody to the M_2 extracellular domain (Zebedee and Lamb, 1989). However, it will be difficult to use the approach of reverse genetics to examine the role of the M_2 protein cytoplasmic tail in virus assembly

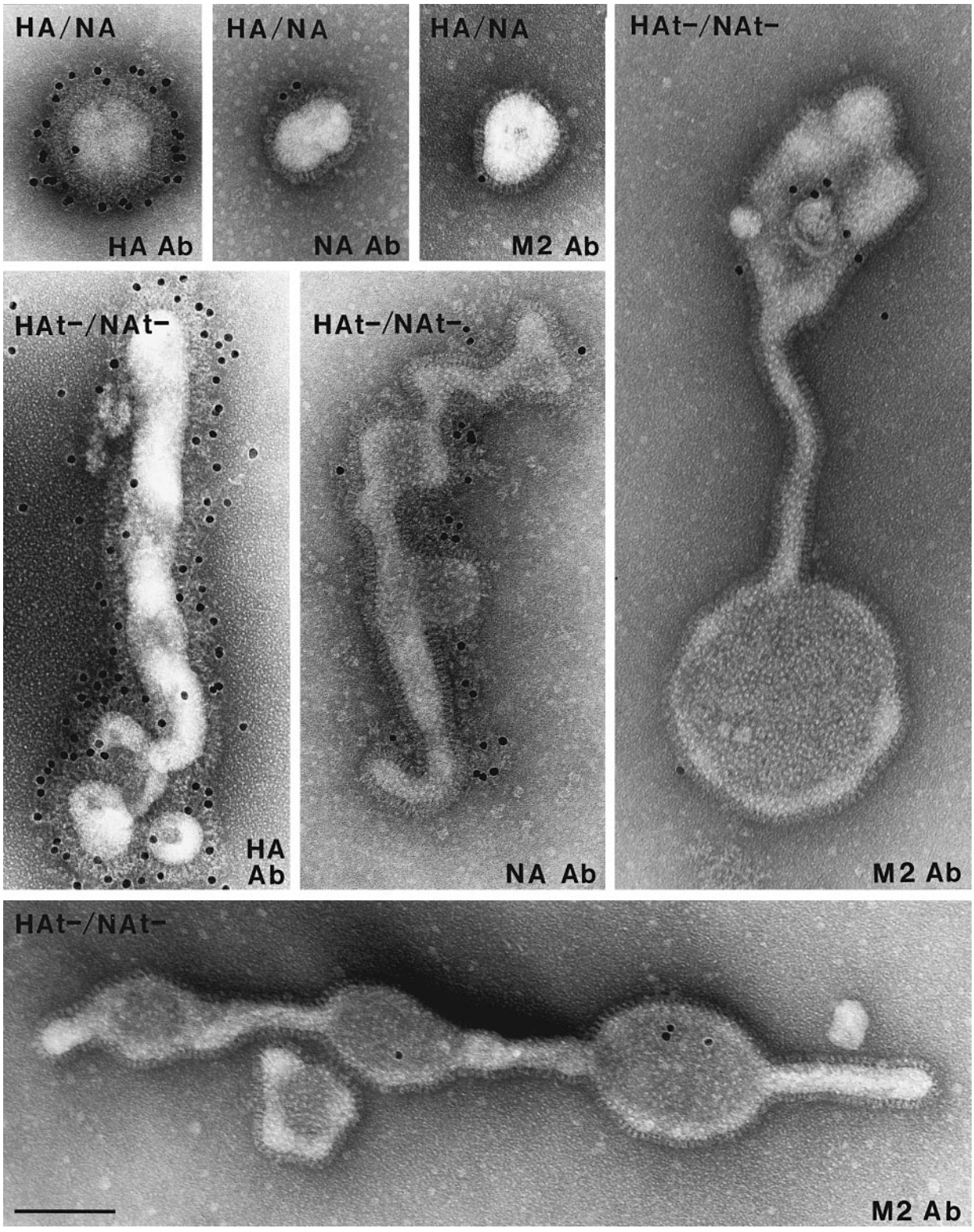

Fig. 6. Immunoelectron microscopy of the HA/NA and HAt–/NAt– viruses. Virions were grown in embryonated eggs, purified on rate zonal and equilibrium sucrose density gradients and virions were absorbed onto Ni grids and incubated with HA-specific mAb D6/1 (HA), NA-specific polyclonal goat serum (NA) or M_2-specific mAb 14C2 (M2). Grids were washed in TBS and stained with goat anti-mouse IgG coupled to 10 nm colloidal gold or donkey anti-goat IgG coupled to 10 nm colloidal gold. Prior to examination, virions were negatively stained with phosphotungstic acid. Virus designations are as indicated in the legend to Figure 2. Bar = 80 nm.

as it has been found that removal of a single C-terminal residue from the M_2 protein prevents rescue of infectious virus (Castrucci and Kawaoka, 1995).

A large population of M_1 protein molecules associates with cellular membranes shortly after synthesis, and this membrane association is an intrinsic property of the M_1 protein (Hay, 1974; Kretzschmar *et al.*, 1996; Zhang and Lamb, 1996). However, biochemical evidence for the

putative interactions of the M_1 protein with the HA or NA cytoplasmic tails has been difficult to obtain, perhaps because these putative interactions are usually too weak to survive experimental fractionation protocols (Kretzschmar *et al.*, 1996; Zhang and Lamb, 1996). None the less, by using vaccinia virus recombinants that express high levels of HA, NA and M_1 proteins, some evidence has been obtained that suggests the HA and NA cytoplasmic tails cause an increased association of the M_1 protein with membranes (Enami and Enami, 1996). It has been proposed that the M_1 protein of influenza virus (and similarly for other enveloped viruses that have a matrix protein) provides the ability to bend membranes from the inside ('push') and that bending of membranes from the outside ('pull') is provided by the cytoplasmic tails of HA and NA (Simons and Garoff, 1980; Mebatsion *et al.*, 1996). If this is the case, it provides an explanation for the poor budding efficiency of HAt–/NAt–. Furthermore, for influenza virus, the irregular morphology of the HAt–/NAt– virions suggests that the normal 'pull' of the cytoplasmic tails provides a driving force to pinch off virions. One scenario for efficient budding is that the membrane-associated M_1 protein forms an interaction with the cytoplasmic tails of the integral membrane proteins and this M_1 protein–integral membrane protein complex acts as a nucleation point to attract cytoplasmic M_1 protein–RNP complexes (Whittaker *et al.*, 1996). The two complexes would be brought together through the formation of M_1 protein–M_1 protein interactions. We suggest from the data presented here that for the normal budding of influenza virus, the requirements for glycoprotein cytoplasmic tail interactions with an internal virion component (probably the M_1 protein) are so critical that these cytoplasmic tail interactions are dually redundant. It is proposed that the M_1 protein has two domains for the interaction of the HA or NA cytoplasmic tail. The interaction of the NA cytoplasmic tail is more important than the interaction of the HA cytoplasmic tail for spherical particle formation, but when both interactions are ablated, budding is inefficient and very irregular shaped particles are formed.

Materials and methods

Cells and viruses

MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. A rescued influenza virus (transfectant) which contains NA lacking its cytoplasmic tail, was kindly provided by Peter Palese (García-Sastre and Palese, 1995) and here is designated NAt–. This virus is derived from subtype A/WSN/33 (H1N1) and the NAt– transfectant virus is in a WSN genetic background. The influenza virus transfectant which contains HA derived from subtype A/Udorn/72 (H3N2) in a WSN genetic background, and here is designated HA/NA, has been described previously (Jin *et al.*, 1996). An influenza virus transfectant which contains HA lacking its cytoplasmic tail (by insertion in the cDNA of three consecutive stop codons) and which also contains a substitution of Cys555 for methionine previously was called Mtr (Jin *et al.*, 1996) and here is designated HAt–/NA. Plaque assays and tissue culture endpoint dilution assays were performed as described previously (Paterson and Lamb, 1993).

Isolation of the HAt– and NAt– double cytoplasmic tail deletion virus

To isolate an influenza virus reassortant containing HA or HAt– (H3 subtype) and NA or NAt– (N1 subtype), the HA and HAt– first had to be removed from their existing WSN genetic background and transferred to a genetic background of subtype A/Japan/305/57 (H2N2) by mixed infection of UV-irradiated HAt–/NA and A/Japan/305/57 using established procedures (Schulman and Palese, 1976). The reassortants obtained were designated HA/Japan and HAt–/Japan (H3N2). Plaque-purified viruses were amplified in the allantoic cavity of embryonated chicken eggs. These viruses were then used to make a new reassortant with the NAt– virus. Briefly, allantoic fluid stocks of HA/Japan and HAt–/Japan were exposed to UV irradiation (Sylvania Germicidal 15 W) for 30 s at a distance of 30 cm. Embryonated eggs were then inoculated with a mixture of the UV-irradiated viruses and 10^5 p.f.u. of non-irradiated NAt– virus. After overnight incubation, the allantoic fluid containing virus was harvested, mixed with DMEM/1% bovine serum albumin containing goat antibodies specific for H1 HA and N2 NA (Influenza Virus Repository, National Institutes of Allergy and Infectious Diseases) for 60 min at 4°C and the mixture re-inoculated into eggs at various dilutions. The eggs were then incubated for 72 h at 36°C and the allantoic fluid harvested as a virus inoculum. Reassortant viruses were serially diluted and used to infect MDCK cells in an end-point dilution assay. $TCID_{50}$ values were calculated according to the method of Reed and Muench (1938). Tissue culture supernatants from the end-point were biologically cloned by plaque passage in MDCK cells. The parental origins of the HA and NA of the reassortant viruses were determined by the distinct electrophoretic mobility of their polypeptides and by their reactivity with subtype-specific antibodies (see below). Virus stocks were amplified in 11-day-old embryonated chicken eggs.

Virus purification

Virions grown in embryonated eggs were first purified on 20–60% sucrose density gradients and subjected to rate zonal centrifugation as described previously (Paterson and Lamb, 1993). Virions were further purified on 15–65% sucrose gradients containing D_2O/NTE [0.1 M NaCl, 0.01 M Tris–HCl (pH 7.4), 0.001 M EDTA]. Fractions were collected from the gradients and each fraction subjected to hemagglutination assay and to SDS–PAGE.

Metabolic labeling of virus-infected cells, immunoprecipitation, immunoblotting and SDS–PAGE

Monolayers of MDCK cells were infected with influenza viruses at a m.o.i. of 5–10 p.fu./cell, and, at 4 h post-infection, cultures were incubated for 30 min with DMEM deficient in methionine and cysteine (DME met^- cys^-). The cells were metabolically labeled with Tran[^{35}S]-label for 3 h in DME met^- cys^- and cells lysed in RIPA buffer (Lamb *et al.*, 1978). Lysates were clarified by centrifugation (55 000 r.p.m. for 10 min in a Beckman TLA.100.2 rotor) prior to immunoprecipitation using appropriate sera. TPCK-trypsin digestion of HA in cell lysates and analysis of polypeptides by SDS–PAGE were performed as described previously (Jin *et al.*, 1994). Immunoblotting was performed by transferring polypeptides from a gel to a PVDF membrane (Immobilon-P membranes, Millipore) using a semi-dry transfer system (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The membrane was blocked with Blotto [5% dry milk in PBST (0.3% Tween 20 in phosphate-buffered saline)] for 1 h. Following three successive washes with PBST, the blot was incubated for 60 min with goat polyclonal antiserum to H3 HA, N1 NA or N2 NA (Influenza Virus Repository, National Institute of Allergy and Infectious Diseases) or M_2-specific mAb 14C2 (Zebedee and Lamb, 1988), with all antibodies diluted 1/1000 in Blotto. After washing as above, the blot was then incubated for 60 min with peroxidase-conjugated rabbit anti-goat or rabbit anti-mouse IgG (Cappel) diluted 1/1000 in Blotto. The blot was washed as before, stained with an ECL Western blot detection kit (Amersham, Arlington Heights, IL) for 1 min and exposed to X-ray film.

RNA extraction, reverse transcription and PCR

Virion RNA was extracted from egg allantoic fluids by using SDS/phenol and phenol/chloroform, transcribed by reverse transcriptase and cDNA amplified by PCR. For A/WSN/33 NA (N1), oligonucleotide N1NA5′ GAGAAGCTTAGCAAAAGCAGGAGTTTAA was used in the reverse transcription reaction and PCR was performed with N1NA5′ and N1NA519, an oligonucleotide corresponding to the WSN NA gene nucleotides 519–536. The oligonucleotide N2NA5′ AGCAAAAGCAGGAGTGA was used in the reverse transcription of N2 RNA and the cDNA amplified by N2NA5′ and N2NA282, an oligonucleotide corresponding to the A/Japan/305/57 NA gene nucleotides 282–299. The nucleotide sequence of the NA PCR DNA was determined as described previously (Jin *et al.*, 1994).

NA activity determination

The neuraminidase activity of purified virus was determined as described previously (Paterson and Lamb, 1987). Briefly, 5 μg of virus protein in

duplicate was mixed with 0.2 M sodium phosphate buffer, pH 5.9, containing 1 mM acetylneuraminyl lactose (Sigma) as substrate in a total volume of 200 µl, and the reactions were incubated at 37°C for 3 h. Then 100 µl of 0.05 M periodate (pH 1.0) was added and the incubations continued for 30 min at 37°C. Finally, 800 µl of 2% (w/v) sodium arsenite was added and the samples boiled for 10 min. The chromophore was extracted with acid–butanol (1:20 HCl:butanol) and the A_{549} determined using a spectrophotometer. The NA activity was expressed as the amount (µg) of the *N*-acetylneuraminic acid released per µg viral protein.

Electron microscopy and immunocytochemistry

The procedure for negative staining and immunostaining purified virus was modified from that of Murti (Murti *et al*., 1985; Murti and Webster, 1986). Briefly, purified transfectant viruses were allowed to absorb onto parlodion-coated nickel grids for 30 s. Grids were floated on a drop of Tris-buffered saline (TBS), pH 7.4, for 5 min, after which they were blocked by floating on drops of 3% ovalbumin in TBS for 1 h. Grids were washed with TBS for 5 min and then incubated for 1 h with goat NA-specific polyclonal sera, HA-specific mouse mAb ascites fluid D6/1 or M_2-specific mouse mAb ascites fluid 14C2 diluted 1/300 in 1% ovalbumin in TBS. Following three successive washes with TBS for 10 min each, samples were incubated for 1 h with either donkey anti-goat IgG coupled to 10 nm gold particles or goat anti-mouse IgG coupled to 10 nm gold particles diluted 1/10 in 1% ovalbumin in TBS, as appropriate. Colloidal gold and gold–antibody conjugates were prepared essentially as described by Slot and Geuze (1985). Grids were washed in TBS as above and finally stained with 2% phosphotungstic acid, pH 6.6. Prior to examination, a thin layer of carbon was evaporated onto the grids. Incubations were carried out at room temperature in a humidified chamber and all solutions were filtered before use. For the negatively stained virion preparations, random fields of view were photographed at low magnification before quantification of the length and diameter of the particles.

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