Formation of virus-like particles from human cell lines exclusively expressing influenza neuraminidase

Jimmy C. C. Lai,1 Wallace W. L. Chan,1 François Kien,1 John M. Nicholls,2 J. S. Malik Peiris1,3 and Jean-Michel Garcia1

Correspondence
Jean-Michel Garcia
jmgarcia@hku.hk

1HKU-Pasteur Research Centre, Hong Kong SAR
2Department of Pathology, The University of Hong Kong, Hong Kong SAR
3Department of Microbiology, The University of Hong Kong, Hong Kong SAR

The minimal virus requirements for the generation of influenza virus-like particle (VLP) assembly and budding were reassessed. Using neuraminidase (NA) from the H5N1 and H1N1 subtypes, it was found that the expression of NA alone was sufficient to generate and release VLPs. Biochemical and functional characterization of the NA-containing VLPs demonstrated that they were morphologically similar to influenza virions. The NA oligomerization was comparable to that of the live virus, and the enzymic activity, whilst not required for the release of NA-VLPs, was preserved. Together, these findings indicate that NA plays a key role in virus budding and morphogenesis, and demonstrate that NA-VLPs represent a useful tool in influenza research.

INTRODUCTION

Influenza A viruses are lipid-enveloped members of the family Orthomyxoviridae. They contain eight negative-sense, single-stranded RNA segments encoding ten viral proteins. Influenza virions are pleomorphic, although generally their shape is roughly spherical with a diameter of <150 nm. However, larger (100–400 nm) influenza virions are also generated, as are filamentous forms (Fujiyoshi et al., 1994).

Influenza viruses derive their lipid envelope by budding from the plasma membrane of infected cells, and progeny virions are normally not found inside the host cell. Therefore, assembly and budding are the final but essential steps in the virus life cycle. The M1 matrix protein is the most abundant protein in the influenza virion and plays a critical role in both virus assembly and budding (reviewed by Nayak et al., 2009). M1 affects virus assembly by interacting with the core viral ribonucleocapsid (vRNP) and cytoplasmic tail of transmembrane proteins, forming a bridge between the two layers, as well as recruiting the internal viral proteins and viral RNA to the plasma membrane in a cooperative manner (Noda et al., 2006). In addition, M1 interacts with the lipid bilayer producing an outward bending of the membrane, and this has been postulated to be the major driving force of influenza budding, as cells expressing M1 protein alone produce virus-like particles (VLPs) (Gómez-Puertas et al., 2000; Latham & Galaeza, 2001).

Haemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins in the influenza viral membrane. HA binds to sialic acid receptors on the cell surface and mediates the fusion process (Matrosovich et al., 2006), whereas NA cleaves the terminal sialic acids from the cell-surface glycans to facilitate release of the progeny virus from the host cell and prevent aggregation of virus particles (Bucher & Palese, 1975; Air & Laver, 1989). NA is also essential in the initial stage of virus infection by enhancing HA-mediated fusion (Su et al., 2009), helping the virus to penetrate the mucin barrier protecting the airway epithelium (Matrosovich et al., 2004) and promoting virus entry (Ohuchi et al., 2006). Studies of mutant influenza viruses have previously shown that the cytoplasmic tails of HA and NA contribute to control virus assembly (Zhang et al., 2000) and virus morphology (Jin et al., 1997). The specific role of HA and NA in virus budding is controversial. COS-1 cells expressing HA alone did not give rise to VLP production (Gómez-Puertas et al., 2000); however, assembly and budding of a NA-deficient virus mutant could be rescued with exogenous bacterial NA to the level of wild-type virus (Liu et al., 1995). These studies suggested that the presence of both viral proteins is not essential for virus budding. Nevertheless, studies of cells expressing recombinant viral proteins from an H3N2 virus demonstrated that both HA and NA were involved in the budding process (Chen et al., 2007).

In this study, using a plasmid-driven VLP production system in human embryonic kidney (HEK-293T) cells, we looked at the minimal virus requirements for influenza virus assembly and budding. We showed that both H5 HA and N1 NA could be the driving forces for virus budding, whereas M1 had only a limited contribution. In addition, we demonstrated that expression of NA alone could lead to

Supplementary figures are available with the online version of this paper.
the budding of particles. The influenza VLPs formed from NA (NA-VLPs) were morphologically similar to influenza virions and had an intact sialidase enzymic activity. Comparable results in generating NA-VLPs were found with NA from both seasonal and pandemic H1N1 and avian H5N1 viruses.

RESULTS

Determination of minimal virus requirements for VLP formation and release

In order to determine which viral structural protein(s) are essential for VLP formation and release, HEK-293T cells were transfected with plasmids encoding H5N1 proteins HA, NA and M1, singly or in combination. The protein composition of the VLPs released into the culture medium was analysed by Western blotting (Fig. 1a), and budding of VLPs from the transfected cells was visualized by transmission electron microscopy (TEM) (Fig. 2). None of the transfections affected the protein expression system, as indicated by analysis of expression of the GAPDH housekeeping protein. Co-transfection of genes encoding different viral proteins had a minimal impact on individual expression levels, as shown in cell lysates (Fig. 1a).

Expression of HA, NA and M1 in the cytosol appeared to be comparable. Release was quantified by densitometric analysis of Western blots and was expressed as the percentage of overall production (cumulative from lysates and supernatant). VLPs were not detected by Western blotting in the culture medium of cells expressing HA alone unless exogenous bacterial NA was added (Fig. 1a, lanes 1 and 2), suggesting that VLPs budding from HA-expressing cells could not be released without exogenous NA, as confirmed by VLP aggregation on the cell surface in TEM (compare Fig. 2a and c). When M1 was expressed alone, a small amount of the protein was detected in the supernatant and no significant change was observed following the addition of exogenous NA (Fig. 1a, lanes 4 and 5). However, we were not able to detect VLP structures from cells expressing M1 alone (Fig. 2d). When HA and M1 were co-expressed, VLPs were released into the supernatant only in the presence of exogenous bacterial NA (Fig. 1a, lanes 7 and 8) with an increase in HA and M1 content when compared with their individual expression. Interestingly, the release of both HA and M1 was significantly enhanced by the co-expression of NA (Fig. 1a, lanes 3, 6 and 9). In addition, VLPs containing NA were easily detected from cells expressing NA exclusively (Fig. 1a, lane 10) and could be widely observed by TEM (Fig. 2b, f). These results confirmed the
requirement of NA enzymic activity for HA-containing VLP release, but also suggested a role of NA in VLP budding. Comparison of protein expression in the cell lysates as well as in the supernatant of infected or HA/NA/M1-transfected cells showed that HA and NA had similar expression levels (Fig. 1b); however, the amount of M1 released with the budding VLPs in the supernatant was approximately half that found in virus-infected cells. This is compatible with previous reports that M1 requires interaction with other viral proteins, such as the structural proteins HA/NA (Wang et al., 2010), as well as with vRNPs, which are absent from VLPs. Using TEM, the VLPs were seen to be pleomorphic, with spherical or filamentous forms (Fig. 2b, f), similar to influenza virions.

**Physical and functional characterization of NA-VLPs produced in cells expressing influenza virus NA alone**

NAs from the H5N1 subtype and from seasonal and pandemic H1N1 subtypes were included in this study. The culture medium of cells expressing NA alone was harvested for detection of released particles. FLAG-tagged NAs were used to allow detection of the protein from different viral origins with anti-FLAG antibodies at the same antibody-binding affinity. No difference in VLP formation efficiency was observed between tagged and non-tagged NA (data not shown). Furthermore, similar particles were seen by TEM for all three subtypes studied (see Supplementary Fig. S1, available in JGV Online). Sucrose gradient (20–60 %) centrifugation analysis demonstrated the presence of NA-VLPs in the intermediate pellet fractions between 30 and 50 % sucrose (Fig. 3b–d), which was similar to the result observed with influenza A/WSN/33 (H1N1) virions.

![Fig. 2. TEM of cells expressing HA, NA and M1 from A/Cambodia/JP52a/2005 (H5N1). HEK-293T cells were processed for TEM at 36 h post-transfection. VLP budding was observed in cells expressing HA without exogenous NA treatment (a, e) or with the addition of exogenous NA (exoNA; c) or when NA was expressed alone (b, f), but not when M1 was expressed alone (d). Arrowheads in (a) indicated a VLP (A) and a filopodium (B).](image)

![Fig. 3. Physical and functional characterization of NA-VLPs. (a–e) Western blot analysis of fractions from the sucrose gradient from 20 % (fraction 1) to 60 % (fraction 20) sucrose of A/WSN/33 (H1N1) virions collected from infected MDCK cells (a), from the supernatant of HEK-293T cells transfected with NA plasmid from seasonal H1N1 (b), pandemic H1N1 (c) or highly pathogenic avian influenza (HPAI) H5N1 (d), or from immunopurified N1 protein (e). (f) The NA enzymic activity of the corresponding sucrose fractions was tested using a NA-Star chemoluminescent assay and expressed in arbitrary units (AU) defined as the percentage of the fraction with the highest signal.](image)
In contrast, purified NA proteins were only present in the lighter fractions (~20% sucrose) of the gradient (Fig. 3e). In addition, NA proteins that were purified or on the VLP surface or from the virus were functionally active, as shown using a NA activity assay (Fig. 3f). The apparent shift of one fraction towards a higher concentration of sucrose for the virus versus the VLP was probably due to the presence of nucleic acid in the virus (RNPs), slightly decreasing their buoyancy.

Kinetic analysis of NA-VLP production showed that it was continuous for at least 60 h post-transfection, with continuous accumulation of the NA-VLP in the supernatant (Fig. 4a) when using NA from either pandemic H1N1 or H5N1. Cleavage of caspase-3 was not detected any time point, indicating that there was no apoptosis (Fig. 4b). Electron micrographs of transfected cells also failed to detect features of apoptosis during the course of the experiment.

We found that expression of NA alone (N1 from H5N1) was sufficient to promote the release of NA-VLPs (Fig. 5). A mutation of NA in position 262 (E262D), which eliminates the sialidase activity, as well as treatment with oseltamivir (a sialidase inhibitor), failed to affect VLP formation. As expected, co-expression of HA (H5 from H5N1) increased the release of NA, probably by the formation of heterochimeric VLPs (HA/NA-VLPs), but release of HA-containing VLPs was then dependent on sialidase activity.

Immunoblotting analysis of VLPs showed that NAs on the particle surface were in multimeric form, and composed the monomer (~55 kDa), dimer (~110 kDa), trimer (~165 kDa) and tetramer (~220 kDa) (Fig. 6). Multimeric NA complexes on the VLPs were denatured to the monomer by heating in SDS loading buffer with chaotropic agent (urea) or reducing agent (DTT) (Fig. 6, lanes 1–3). Pre-treatment with the cross-linker 3,3'-dithio-bis(sulfo-succinimidylpropionate) (DTSSP) before the denaturing step resulted in multimeric NA complexes being protected from SDS and urea, and partially from DTT (Fig. 6, lanes 4–6). Similar multimeric NA complexes were detected in the H1N1 virions, but not in the samples pre-treated with DTSSP (lanes 7 and 8), probably due to the inability of the antibody to recognize cross-linked antigens. Samples from highly pathogenic H5N1 virus could not be analysed, because protocols for inactivating the infectivity of the highly pathogenic H5N1 virus so that these preparations could be taken out of the Biosafety Level 3 (BSL-3) containment area for further analysis compromised oligomer integrity.

Desialylation of cell-surface sialic acids in cells expressing NA was then studied by lectin binding (see Supplementary Fig. S2, available in JGV Online). A weak binding of Sambucus nigra agglutinin (SNA) was observed in HEK-293T cells, indicating a low level of α-2,6-linked sialic acid on the 293T cells, and this disappeared in cells expressing any of the NAs studied. A similar loss of cell-surface sialic acids was seen in NA-VLP-expressing A549 or Madin-Darby canine kidney (MDCK) cells, which express more α-2,6-linked sialic acid (data not shown). Maackia amurensis agglutinin (MAA) binding confirmed the presence of α-2,3-linked sialic acid on mock-transfected 293T cells, and this was significantly decreased in NA-expressing cells. Cleavage of sialic acids by sialidase is expected to expose the underlying galactose–N-acetyl-D-galactosamine (Gal-GalNAc) and this was demonstrated by increased binding of peanut agglutinin (PNA) to NA-expressing cells.
**DISCUSSION**

Influenza assembly, budding and release are the last but important steps in the replication cycle. VLP production assays using different systems have been developed to determine the viral proteins involved in virus budding (Gómez-Puertas et al., 2000; Latham & Galaeza, 2001; Neumann et al., 2000). Here, we investigated the minimal viral components required for assembly and budding of H5N1 influenza virus using a plasmid-driven VLP formation system similar to that described in previous study on H3N2 virus (Chen et al., 2007).

Previous findings about the major viral proteins responsible for virus budding have been contradictory. Gómez-Puertas et al. (2000) suggested that M1 plays the major role in driving virus budding from the cellular membrane, whereas Chen et al. (2007) found that M1 was not essential for the process, but that HA and NA were necessary. A recent publication (Wang et al., 2010) confirmed that M1 by itself fails to form VLPs, probably due to a lack of membrane-targeting signal, and therefore requires interaction with other viral proteins to be incorporated into the budding virions. In our study, although small amounts of M1 were released from cells expressing M1 alone, no budding particles were observed by TEM (Fig. 2d). A similar finding was recently reported in which it was suggested that M1 was only secreted nonspecifically into the supernatant rather than being released in the form of VLPs (Tscherne et al., 2010).

Nevertheless, co-expression of M1 increased the level of both HA and NA incorporated into VLPs (Fig. 1a, lanes 2 and 8, and 6 and 10), suggesting that M1 helps virus budding, probably by pushing the inner side of the cellular membrane.

VLP formation in cells transfected with HA alone or HA/M1 was completely dependent on the addition of exogenous NA. In the absence of exogenous NA, HA-containing VLPs aggregated on the cell surface and were not released into the medium (Figs 1 and 2). Exogenous NA cleaved the cell-surface sialic acids, allowing the VLPs to be released, and therefore both HA and M1 became detectable in the medium. These results suggested that the expression of HA could provide a driving force to trigger the production of VLPs, although exogenous NA was necessary for the release of the particles.
Previously, it has been suggested that NA is important for virus morphogenesis. Studies using influenza virus lacking cytoplasmic tails of HA or NA demonstrated that virus particles lacking the cytoplasmic tails of NA had an elongated morphology but virus lacking the cytoplasmic tails of HA did not (Jin et al., 1997). Introduction of mutations in both the transmembrane and cytoplasmic domains of NA confirmed that NA is critical to control the virus shape, size and titre (Barman et al., 2004). Studies of a mutant virus containing a large internal deletion in the NA gene showed that the mutant could assemble and bud similarly to wild-type virus (Liu et al., 1995). However, our data have shown for the first time that expression of NA alone in HEK-293T cells could provide a driving force for the formation of extracellular NA-containing particles, and this finding was seen with NA from different influenza viruses of the N1 subtype and demonstrated in at least two additional human cell lines (A549 and HeLa, data not shown). It is also important to note that the release of M1 and HA was greatly enhanced by their co-expression with NA to a level that was not acquired by a high concentration of exogenous NA, indicating that NA is likely to be a major force in driving virus budding. However, whereas Chen et al. (2007) reported that only small amounts of NA were released when this glycoprotein was expressed on its own, our data showed that a large amount of NA was released. This discrepancy is probably due to the difference in influenza virus subtype used in the two studies (N2 in Chen et al., 2007, compared with N1 in this study). In fact, we did observe that N2-subtype NA was expressed at a much lower level than N1 subtype, and that N1 from human seasonal H1N1 was expressed less than the highly pathogenic avian H5N1 (see Supplementary Fig. S3, available in JGV Online).

Furthermore, using a potent sialidase inhibitor and a point mutation (E262D) in NA that inactivates the catalytic site of the sialidase (Huang et al., 2008), we showed that NA activity was not necessary for the release of NA-VLPs in cells (Fig. 5).

To characterize the NA-VLPs, biochemical, physical, morphological and functional studies were carried out, including a sucrose gradient flotation profile, NA functional assay, detection of multimeric NA and a TEM study, as well as desialylation of surface sialic acid on cells producing NA-VLPs. The presence of both influenza virions and NA-VLPs in the middle fractions of the sucrose gradient (Fig. 3a and b–d, respectively) indicated that VLPs have a buoyant density equivalent to native influenza viruses. The enzymic activity assay showed that NAs on the particle surface were functionally active and that the activity was proportional to the amount of protein (Fig. 3f). The morphology of the VLPs was found to resemble that of influenza virions. Most of the VLPs were spherical, although elongated particles were also detected (Fig. 2b and f). No morphological difference was observed in NA-VLPs from high pathogenic avian influenza H5N1, seasonal H1N1, and pandemic swine-origin H1N1 (Supplementary Fig. S1).

It is conceivable that exosomes or vesicles arising from apoptotic bodies may be mistaken for VLPs. However, TEM failed to reveal either apoptosis or the formation of intracellular vesicles (such as the multivesicular bodies of exosomes). Furthermore, we also monitored for apoptosis in transfected cells by attempting to demonstrate the expression and cleavage of caspase-3 in the course of the experiment. Only the uncleaved form of caspase-3 could be detected for the first 60 h post-transfection, suggesting that the VLP-producing cells were not undergoing apoptosis (Fig. 4b). Cross-sections of membrane protrusions such as filopodia (Fig. 2a, arrowhead B) do occur and these may be mistaken for budding of VLPs. However, they can be distinguished from the ‘typical VLP’ by TEM because VLPs (Fig. 2a, arrowhead A) have a more electron-dense outline, which is distinguishable from the cell membrane, except at the budding sites of virus or VLPs (Fig. 2). Taken together, these data indicated that the particles observed by TEM were true VLPs.

NAs on the particle surface were found to be tetrameric in the influenza virions, which is important for some of the NA functions. Using the cross-linker DTSSP, multimeric NAs were protected from urea and we were able to detect multimeric NA complexes comparable to those found on fully infectious H1N1 virions. This result was expected, as maturation of NA has been described to take place in the endoplasmic reticulum where the NA tetramers form before going through the Golgi network to the assembly site at the plasma membrane (Saito et al., 1995). Therefore, budding of NA-VLPs (or virus) from the cell surface would be expected to incorporate only mature tetrameric NA proteins with similar oligomerization to the influenza virus. Failure to detect NA from virions after DTSSP treatment may have been caused by conformational changes of the protein upon conjugation. The cross-linker may also have modified or hidden the residues (such as lysine) involved in the antibody-binding site. Our data indicated that NA on the VLP surface was in oligomeric form, although the technique used did not allow us to quantify the ratio between the monomer, dimer, trimer and tetramer (Fig. 6).

All three neuraminidases included in the study were able to cleave cell-surface sialic acids in both α-2,3 and α-2,6 linkage. As the technique used was qualitative rather than quantitative, we could not quantify a preference in the cleavage activity towards sialic acid with an α-2,3 linkage (Supplementary Fig. S2), as mentioned in previous findings using H1N1 and H3N2 virus NA (Mochalova et al., 2007; França de Barros et al., 2003). We detected a significant increase in β-Gal-GalNac in NA-expressing cells by PNA binding. This supports the role of NA enzymic activity during the initial stage of infection by helping the virus to penetrate through the mucus layer where sialic acid is mainly linked to Gal-GalNac in an α-2,3 configuration.

NA is an important viral component of influenza viruses and the most effective anti-influenza drugs on the market.
are NA inhibitors. Although NA has been studied for decades, most of the data were obtained from purified virions, which require access to a high-level biocontainment laboratory when dealing with highly pathogenic avian influenza viruses. Purified NA proteins have also been widely used for NA study, but these isolated NAs may not have the same properties as NA on the viral surface. It was, in fact, reported that substrate specificities of sialidase activity from purified NA and their original viruses are not identical (Nagai et al., 1995). The NA-VLPs produced in our study, although non-infectious, were multivalent, antigenic, enzymically active and morphologically similar to native influenza viruses. Therefore, they could be a preferable alternative to recombinant NA peptides or proteins as an immunogen to produce antibodies against NA or as antigens for ELISA-type assays. In addition, because the NAs on the VLP surface were not only functionally active but also presented in a multimeric form, these VLPs are a useful tool to investigate NA interactions with host cells, as well as NA inhibitor drugs.

METHODS

Plasmids. Plasmids pcDNA-HA, pcDNA-NA(H5) and pcDNA-M1 corresponding, respectively, to DNA sequences of HA (GenBank accession no. EF456805), NA (GenBank accession no. EF456793) from influenza A/Cambodia/IP52a/2005 (H5N1) and M1 (GenBank accession no. AF144306) from influenza A/Goose/Guangdong/1/96 (H5N1) were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). Both non-tagged and FLAG-tagged HA and NA proteins, tagged at the C-terminus, were used. For the study of NA from different viral subtypes, plasmids pcDNA-NA(H1) and pcDNA-NA(pdmH1) encoding the FLAG-tagged NA of influenza A/Gansu/Chenguan/1129/2007 (seasonal H1N1; GenBank accession no. EU879064) and A/California/04/2009 (swine-origin pandemic H1N1; GenBank accession no. FJ969517), respectively, were prepared. Enzymically inactive pcDNA-NA(E262D) was prepared from pcDNA-NA(H5) by point mutation using a QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions and post-sequenceing selection of clones based on a NA-Star chemiluminescent assay (Applied Biosystems).

Cells and viruses. HEK-293T and MDCK cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO2. Influenza A/WSN/33 (H1N1) virus was propagated in MDCK cells with culture medium supplemented with 0.5 μg phenylalanyl chloromethyl ketone-treated trypsin ml-1. Highly pathogenic avian influenza A/Hong Kong/483/97 H5N1 virus was propagated in MDCK in minimal essential medium.

Antibodies and Western blotting. Vaccinated chicken sera containing anti-HA antibodies (haemagglutination inhibition titre >256) and polyclonal antibodies from rabbit hyperimmunized with avian influenza A virus NA (Abcam) were used to detect non-tagged HA and NA proteins, respectively. Anti-FLAG mouse monoclonal antibody (mAb) (Sigma) was used to detect FLAG-tagged HA and NA. Mouse mAb (clone G2B2; Abcam) was used for the detection of influenza M1 protein. For Western blotting, samples were resolved by 4–12% SDS-PAGE (Invitrogen), electrophoretically transferred onto PVDF transfer membrane, hybridized with appropriate antibodies and detected with ECL Western Blotting Detection Reagent (Amersham). Protein size was estimated using Novex Sharp Pre-stained Protein Standards (Invitrogen). The fraction of protein released in the supernatant was expressed as a percentage of overall expressed protein both in lysates and in supernatant adjusted to the respective volumes followed by normalization relative to GAPDH housekeeping protein expression detected with anti-GAPDH mouse mAb (clone 6C5; Abcam). Quantification was performed by densitometry of scanned images obtained using Scion Image Beta 4.0.3 (Scion Corporation). Each quantification was associated with a calibration curve using a range of FLAG–bacterial alkaline phosphatase proteins (FLAG–BAP; Sigma) covering the range of concentrations measured. The exposure times were chosen in order to quantify the signal intensity within the linear range of the calibration curve below saturation of the signal, as monitored by the image analysis.

Detection of VLP production in cells expressing influenza HA, NA and M1 proteins. HEK-293T cells were transfected with different combinations of plasmids using a CalPhos Mammalian Transfection kit (Clontech) according to the manufacturer’s instructions. The amount of plasmids pcDNA-HA, pcDNA-NA(H5) and pcDNA-M1 in the transfection mixture was 5 μg each, and empty vector was used to replace the omitted plasmids as appropriate. At 12 h post-transfection, the medium was replaced with fresh DMEM with 10% FBS, and exogenous bacterial NA (Vibrio cholerae, 6.25 mU ml-1; Roche) was added to the medium as appropriate. At 60 h post-transfection, the supernatant was collected and cell debris was removed by filtration through a 0.45 μm filter. Filtered culture medium was then layered onto a 30% sucrose/HEPES buffer (2 mM HEPES (pH 7.4), 125 mM NaCl, 0.9 mM CaCl2, 0.5 mM MgCl2) cushion and centrifuged at 28000 r.p.m. for 2.5 h at 4 °C in a Beckman SW32 rotor and the pellet resuspended in HEPES buffer. Cell lysates were collected at the same time by the addition of lysis buffer [20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton-X, protease inhibitor mixture (Roche Diagnostics)], followed by centrifugation at 16000 g for 10 min in a bench-top centrifuge. Samples were mixed with 4× LDS sample buffer (Invitrogen) and DTT (100 mM), boiled for 8 min and analysed by Western blotting as described above.

H5N1 virion preparation and analysis. MDCK cells, plated the day before, were inoculated for 1 h at 37 °C with H5N1 virus at an m.o.i. of 1. After washing with PBS, infected cells were incubated at 37 °C overnight. At 24 h post-infection, the supernatant was harvested, clarified by centrifugation before concentration on a 100 kDa pore filter Amicon system (Millipore) and finally resuspended in lysis buffer [25 mM HEPES (pH 7.4), 50 mM KCl, 1% NP-40, 1 mM DTT, protease inhibitor mixture (Roche Diagnostics)]. Cell lysates were also collected after addition of lysis buffer to the infected cells. In each sample, loading buffer [prepared from 6× stock: 62.5 mM Tris/HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromphenol blue, 5% β-mercaptoethanol] was added and boiled for 10 min so that the samples could be taken out of the BSL-3 laboratory.

NA-VLP and H1N1 virion preparation and analysis. For NA-VLP production, HEK-293T, HeLa and A549 cells were transfected with 10 μg pcDNA-NA(H5), pcDNA-NA(H1) or pcDNA-NA(pdmH1) as indicated above. Transfection with an equal amount of empty vector was carried out as a mock control. After ultracentrifugation of clarified supernatant as described above, the pellet was resuspended in NTE [10 mM Tris/HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA], loaded onto a sucrose gradient (20–60%) and centrifuged at 35000 r.p.m. for 2 h at 4 °C in a Beckman SW41 rotor. Twenty fractions were collected manually from the top to the bottom.

Influenza A/WSN/33 (H1N1) virions were purified from infected MDCK cultures at 72 h post-infection. The supernatant was harvested and clarified by centrifugation at 2000 g for 15 min. Virions were then pelleted, loaded onto a sucrose gradient (20–60%)...
and centrifuged as described above for VLPs. Recombinant NA proteins purified from transfected cell lysates by immunoprecipitation using anti-FLAG M2–agarose affinity gel (Sigma) were layered onto a sucrose gradient as above, as an additional control.

All the above-mentioned sucrose fractions of VLPs, virions and purified proteins were boiled in LDS loading buffer with DTT and analysed by Western blotting (see above). The sialidase activities of the NA-VLPs and purified proteins were measured using a NA-Star chemoluminescent assay. Oligomerization of NA on the VLP surface was studied using the cross-linker DTSSP (Thermo Scientific). NA-VLPs were mixed with DTSSP (5 mM) to stabilize the multimeric NA complexes prior to the addition of LDS loading buffer, followed by addition of urea (8 M) or DTT (100 mM) as appropriate. DTSSP-pre-treated VLPs were analysed directly by Western blotting, as described above.

**TEM.** Transfected HEK-293T cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide. The fixed cells were then pelleted in 1% agar, dehydrated in increasing concentrations of ethanol and embedded in epoxy resin. Ultrathin sections of the samples were visualized in a Philips EM208s electron microscope after staining with 2% aqueous uranyl acetate and Reynold’s lead citrate.

**Kinetics of NA-VLP release.** HEK-293T cells were transfected with pcDNA-NA(H5) or pcDNA-NA(pdmH1) as described above. At 12 h post-transfection, the culture medium was replaced with fresh DMEM with 10% FBS. Supernatant and cell lysates were collected at 20, 28, 36 and 60 h post-transfection as described above. Samples were boiled with LDS loading buffer and DTT for 8 min and analysed by Western blotting.

**Effect of NA enzymic activity on the production of VLP.** A single amino acid change (E262D) in the NA is known to eliminate the sialidase activity of the NA molecule (Huang et al., 2008). HEK-293T cells were transfected with pcDNA-NA(H5) or pcDNA-NA(E262D), with or without pcDNA-HA. At 12 h post-transfection, the culture medium was refreshed, and 50 μM oseltamivir carboxylate (a sialidase inhibitor) was added to the medium as indicated. Supernatant and cell lysates were collected at 60 h post-transfection and protein levels in VLPs were analysed by Western blotting. The sialidase activity of the supernatant was measured using a NA-Star chemoluminescent assay.

**Lectin staining for cell-surface sialic acid.** Lectin histochemistry analysis was performed as described previously (Nicholls et al., 2007). Briefly, HEK-293T cell lines stably or transiently expressing N1 from seasonal H1N1 (HuNA) or pandemic H1N1 (pdmNA) or from avian influenza H5N1 (AvNA) were fixed with 4% paraformaldehyde. After washing with PBS, lectin staining was performed with SNA (SNA-I; Roche) and MAA (MAA-II; Vector) to detect α-2,6- or α-2,3-linked sialic acid, respectively. PNA (Roche) was used to identify the terminal Gal-GalNAc. Biotin-conjugated lectins were used with avidin–peroxidase counterstaining (Vector).

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