Assembly of Subtype 1 Influenza Neuraminidase Is Driven by Both the Transmembrane and Head Domains*\textsuperscript{a,b}

Received for publication, October 3, 2012, and in revised form, November 11, 2012 Published, JBC Papers in Press, November 13, 2012, DOI 10.1074/jbc.M112.424150

Diogo V. da Silva\textsuperscript{1,} Johan Nordholm\textsuperscript{1,} Ursula Madjo, Annika Pfeiffer, and Robert Daniels\textsuperscript{2}

From the Department of Biochemistry and Biophysics, Center for Biomembrane Research, Stockholm University, SE-106 91 Stockholm, Sweden

**Background:** Influenza neuraminidase is thought to function as a tetramer, but what drives tetramerization is unknown.

**Results:** The neuraminidase transmembrane domain (TMD) contributes to the assembly process by tethering the stalk to the membrane in a tetrameric conformation.

**Conclusion:** The enzymatic head and TMD coordinate the proper assembly of neuraminidase.

**Significance:** Single-spanning TMDs can contribute to the assembly of distal domains.

Neuraminidase (NA) is one of the two major influenza surface antigens and the main influenza drug target. Although NA has been well characterized and thought to function as a tetramer, the role of the transmembrane domain (TMD) in promoting proper NA assembly has not been systematically studied. Here, we demonstrate that in the absence of the TMD, NA is synthesized and transported in a predominantly inactive state. Substantial activity was rescued by progressive truncations of the stalk domain, suggesting the TMD contributes to NA maturation by tethering the stalk to the membrane. To analyze how the TMD supports NA assembly, the TMD was examined by itself. The NA TMD formed a homotetramer and efficiently trafficked to the plasma membrane, indicating the TMD and enzymatic head domain drive assembly together through matching oligomeric states. In support of this, an unrelated strong oligomeric TMD rescued almost full NA activity, whereas the weak oligomeric mutant of this TMD restored only half of wild type activity. These data illustrate that a large soluble domain can force assembly with a poorly compatible TMD; however, optimal assembly requires coordinated oligomerization between the TMD and the soluble domain.

Proteins are assembled from one or more domains that can function independently, cooperatively, or as part of a complex. Through mutations, domain insertions, or deletions, the function of a protein can be altered by changing its topology, folding, localization, activity, or potential interacting partners (1, 2). RNA viruses exploit all of these processes to create variation and functional diversity within the genomes of their progeny for host adaptation (3–5).

The type A influenza surface antigen neuraminidase (NA)\textsuperscript{3} was initially described as a three domain protein based on its appearance in electron micrographs (6). The largest domain is the enzymatic head, which is tethered to the viral membrane by a filamentous stalk domain connected to an N-terminal transmembrane domain (TMD) (7). The enzymatic head domain has been well characterized and is known to facilitate viral release and prevent interparticle aggregation by removing the cell surface receptor (sialic acid) that HA associates with to initiate viral entry (8–11). The large amount of biochemical and structural data available for NA makes it an ideal substrate to investigate how a TMD contributes to the assembly of a distal ectodomain into a functional enzyme.

Nine different NA subtypes (N1–N9) have been found in type A avian influenza strains and structures have been solved for most of the subtypes, which demonstrate the enzymatic head domain forms a tetramer (12–14). In contrast, no structural data are available for the NA TMD that is part of the N-terminal signal anchor sequence and is known to link the head domain to the viral envelope by a length-variable stalk (15–17). The changes in stalk length are thought to regulate the distance of the enzymatic head domain with respect to the cell surface receptor (18–24). How insertions and deletions occur within the stalk domain without altering the assembly of the head domain is not known.

NA oligomerization occurs rapidly and efficiently within the endoplasmic reticulum, possibly through an N-terminal directed process (25–27). However, the direct role of the TMD in NA assembly has received little attention. This is likely a result of the initial observation that NA head domains retained \~100% of their initial enzymatic activity after they were isolated from influenza particles by proteolytic cleavage within their stalk domain (12, 28).

To investigate how the NA TMD contributes to the assembly of the distal head domain into a functional sialidase, we used a systematic molecular and biochemical approach. Our results demonstrate the TMD assemblies into a tetrameric conformation and is required for NA to acquire sialidase activity but not for its synthesis and trafficking. Removal, membrane tethering, or facilitating the tetramerization of the stalk domain all restored activity to NA, which explained that the TMD functions to tether the stalk to the membrane in a tetrameric conformation. Taken together, these data illustrate how the assem-

\* This work was supported by grants from the Swedish Research Council, Swedish Foundation for Strategic Research, and the Carl Trygger Foundation (to R. D.).

\textsuperscript{a} This article contains supplemental “Methods,” text, Figs. 1–3, and an additional reference.

\textsuperscript{b} Both authors contributed equally to this work.

1 To whom correspondence should be addressed. Tel.: 46-8-162460; Fax: 46-8-153679; E-mail: robertd@dbb.su.se.

2 The abbreviations used are: NA, neuraminidase; TMD, transmembrane domain; ss, signal sequence; BN-PAGE, blue native PAGE; GpA, glycoporphin A; Endo-H, endoglycosidase H; PNGase F, peptide-n-glycosidase F; Di, dimeric, Tri, trimeric; Tet, tetrameric.
bly of the TMD coordinates with that of the ectodomain to promote the proper assembly of a type II membrane protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, and Harvesting**—HEK 293T, HeLa, and MDCK from ATCC were cultured in DMEM with 10% FBS, 100 units/ml penicillin, and streptomycin and maintained at 37 °C in a 5% CO2 humidified incubator. For each transfection, 1.5 μl of plasmid DNA was incubated 20 min with 5 μl of LT-1 (Mirius) in 500 μl of Opti-MEM (Invitrogen). Cells were trypsinized from an ~90% confluent 10-cm dish, sedimented (500 × g, 5 min), resuspended in 12 ml of Opti-MEM 10% FBS, and 1 ml of cells were added to each transfection mixture before seeding on 3.5-cm dishes. 48 h post-transfection, cells were washed with cold PBS, pH 7.4, and harvested by scraping in 150 μl of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% n-dodecyl β-d-maltoside, 10 mM N-ethylmaleimide, 1× protease inhibitor mixture (Sigma)). The whole cell lysates were sonicated on ice for 30 s and sedimented (20,000 × g, 5 min), and the post-nuclear supernatants were retained. For the secreted constructs, cells were cultured in Opti-MEM 1% FBS, and the medium was retained and clarified by sedimentation (20,000 × g, 5 min).

**Plasmids and Constructs**—NA mammalian expression vectors were created by PCR overlap cloning (29) using pCDNA3.1A-Myc-His (Invitrogen) containing full-length NA from influenza A/WSN/33 (H1N1) fused with the C-terminal Myc-His tag. NA-TMs constructs were created by excluding residues 35–38, 35–46, 35–56, and 35–66 from NA-WT. For the secreted NA (ssNA32–453) stalk truncations, the cleavable BiP signal sequence (ss) was fused to residue 32, 39, 47, 57, 67, or 73 in NA (WSN33 numbering). Soluble dimeric, trimeric, and tetrameric GCN4 leucine zipper coiled-coil domains (30) were inserted after the ss cleavage site to create s\textsubscript{Dx/Tx/Ty}\textsubscript{Na32–453}NA1–74, was made by excluding residues 75–453, glycophorin A (GpA) and G83I TMD chimeras involved swapping their 17-residue TMDs with residues 7–24 from NA-WT. The GALLEX constructs were derived from the pBLM-GpA/GpA-G83I plasmids kindly provided by Dirk Schneider (Johannes Gutenberg University Mainz). The indicated TMD test segments were amplified from the related NA construct in pCDNA and fused between LexA and MalE by overlap PCR cloning. Point mutations were created by site-directed mutagenesis, and all constructs were verified by sequencing (Eurofins MWG Operon).

**Blue Native PAGE and Immunoblotting**—Post-nuclear supernatants for blue native PAGE (BN-PAGE) were diluted to 85 μl with ACA buffer (750 mM amino-n-caproic acid, 50 mM Bis-Tris, pH 7.0), 15 μl of 5% G250 Coomassie stain was added, and the samples were incubated 10 min at 4 °C prior to resolving on a 5–15% BN-PAGE gradient gel. A pre-transfer at 15 V for 10 min was performed to remove the Coomassie staining followed by transfer to a PVDF membrane. Denaturing immunoblot samples were diluted in non-reducing or reducing (0.1 M DTT) Laemmli sample buffer, heated to 37 °C for 5 min, separated by SDS-PAGE, and transferred to a PVDF membrane. Membranes were blocked with milk/PBST (3% nonfat dry milk, PBS, pH 7.4, 0.05% Tween 20) for 30 min and processed using standard immunoblotting protocols with a monoclonal mouse anti-Myc antibody (Cell Signaling). Immunoblots were developed with ECL-prime (GE Healthcare), images were acquired with a CCD camera (Fuji) and quantified using Multigauge software (Fujiﬁlm).

**Neuraminidase Activity Assay and Kinetic Analysis**—Sialedase activity was measured using 2’-(4-methylumbelliferyl)-α-d-N-acetylneuraminic acid as described previously (31). Briefly, standardized samples were diluted to equivalent amounts using lysis buffer or medium for secreted samples and brought up to 190 μl in reaction buffer (0.1 mM potassium phosphate, pH 6.0, 1 mM CaCl2). Reactions were initiated by adding 10 μl of 2 mM 2’-(4-methylumbelliferyl)-α-d-N-acetylneuraminic acid, and the activity was monitored at 30-s intervals for 30 min at 37 °C with excitation and emission wavelengths of 365 and 450 nm in a SpectraMax Gemini EM. The rates were determined using a mubellinhibiferyl standard, and these were then normalized for total protein from reducing SDS-PAGE immunoblots to calculate the percent activity.

**Neu5acCO\textsubscript{3} Extraction, Endo-H, and PNGase F Treatments**—Transfected cells were washed with ice-cold PBS, scraped with 150 μl homogenization buffer (PBS pH 7.4, 1 mM EDTA, 0.2 mM sucrose, 2× protease inhibitor mixture), and passed 30 times through a 27-gauge needle. Unbroken cells and nuclei were removed by sedimentation (2000 × g, 10 min, 4 °C). The isolated vesicle and cytosol fraction was incubated 30 min on ice with 800 μl of 0.1 M Na\textsubscript{2}CO\textsubscript{3} (pH 11.3) and then subjected to ultracentrifugation (180000 × g, 20 min). The resulting pellet containing the membrane fraction was resuspended with reducing Laemml sample buffer and the supernatant containing the soluble fraction was precipitated with 20% TCA before dissolving in sample buffer. Endoglycosidase H (Endo-H) and peptide-n-glycosidase F (PNGase F) treatments were performed on 20 μl of transfected cell post-nuclear supernatants according to the manufacturer’s instructions (New England Biolabs) except that 5% SDS was used in place of 10× glycoprotein denaturing buffer (32).

**GALLEX Assay**—SU101 Escherichia coli cells with a constitutively expressed lacZ gene regulated by the LexA operator were kindly provided by Dirk Schneider (Johannes Gutenberg University Mainz) with an established protocol (33). SU101 cells were freshly transformed with each pBLM expression plasmid and grown overnight in the presence of antibiotics. Cultures were back diluted in LB to A\textsubscript{600 nm} = 0.1, induced with 0.05 mM isopropyl 1-thio-β-d-galactopyranoside at 37 °C for 2 h until A\textsubscript{600 nm} = 0.6. β-galactosidase activity was determined as described previously (34).

**Sequence analysis and statistics**—All of the NA influenza A protein sequences were retrieved from the NCBI Influenza Virus Resource Database (http://www.ncbi.nlm.nih.gov/ genomes/FLU/) in April 2012. The TMDs (residues 7–34) were determined using the G predictor for biological hydrophobicity (35). Stalk length was calculated from residue 35 to the first residue not resolved in the available subtype 1 NA crystal structures of the head domain, eight amino acids before the conserved large loop disulfide bond joining the N and C termini (13, 14, 36). Glycosylation frequencies were calculated by the number of NX(S/T) motifs in the unique N1 stalk domains.
All error bars represent one S.D. from three independent experiments.

RESULTS

A Minimal Stalk Length Is Required for Proper NA Assembly—Influenza neuraminidase is composed of an enzymatic head domain connected to a stalk and TMD that is located in the signal anchor sequence (Fig. 1A). Across and within many neuraminidase subtypes (N1–N9), the stalk region has substantial length variation with respect to host and its corresponding hemagglutinin (H1–H16) subtype. To display the stalk length variation within human and avian type A influenza strains. The available N1 sequences were grouped by host and their hemagglutinin (H) subtype, and the number of unique stalks at each length was calculated. C, similarity of NA activity profiles from influenza A/WSN/33 (H1N1) particles and lysates from 293T cells transfected with NA-WT-Myc exposed to EDTA, the reducing agent DTT, elevated temperature, and the detergent DDM. NA-Y386F-Myc is an inactive mutant used as control. D, depiction of the stalk deletions in NA WSN33 that fuse residue 34 with 39, 47, 57, or 67. E, immunoblots of lysates from cells transfected with the indicated NA stalk deletions resolved by non-reducing (NR) and reducing (RD) SDS-PAGE. The percentage of NA-WT-Myc activity for each construct was normalized to the NA-WT levels on reducing blots (n = 3). Note the oxidative aggregates (Oxagg) for NA-TM67–453-Myc. F, oligomeric state of the stalk deletions in NA were determined by BN-PAGE immunoblots.

What allows N1 to undergo stalk insertions or deletions and how do they affect production, trafficking, and activity? To investigate this, a quantitative transfection-based system was established using Myc-tagged N1 from the human H1N1 strain WSN33 (supplemental Fig. 2, A–C). The tag did not impair NA-WT plasma membrane trafficking, or its enzymatic properties, as the sialic acid binding defined by the Michaelis constant (Km) and processing (Vmax) were similar to NA in WSN33 influenza particles (supplemental Fig. 1, A and B). Furthermore, the sialidase activity profiles followed the same trends upon
treatment with EDTA, temperature, DTT, and detergent (Fig. 1 and supplemental Fig. 1, C–E).

Using this system, a series of constructs were analyzed where increasing numbers of residues were removed directly following the TMD at position 34 to examine how stalk deletions affect NA activity (Fig. 1D). The constructs were given the nomenclature NA-TM<sub>X</sub>-453, where X is the residue in NA from WSN33 (either 39, 47, 57, or 67) that was fused to the TMD.

NA-WT, NA-TM<sub>39</sub>-453, and NA-TM<sub>47</sub>-453 had similar activity levels, and all of these constructs resolved as oxidized dimers on nonreducing SDS-PAGE because they possessed Cys-49 in the stalk that forms an intermolecular disulfide bond (Fig. 1E and supplemental Fig. 2B). NA-TM<sub>57</sub>-453, which lacks Cys-49, was observed as an oxidized monomer and possessed ~55% of NA-WT activity. NA-TM<sub>67</sub>-453, which lacks the entire stalk region, showed substantial misfolding as the head domain formed incorrect intramolecular disulfide bonds and aggregated (Ox<sub>Agg</sub>), explaining its lack of activity. On BN-PAGE, all of the constructs possessing sialidase activity predominately resolved as tetramers slightly below the soluble 440 kDa marker (Fig. 1F). Together, these results indicate that NA assembly is not dramatically affected by changes in the stalk and that a minimal stalk length of 10 amino acids from the membrane is needed for the head domain to fold.

Production of Enzymatically Active NA Is Impaired in the Absence of the TMD—To address the contribution of the NA TMD to the production of functional NA, residues 32–453 were attached to a cleavable signal sequence (ss) (Fig. 2A). ssNA<sub>32</sub>–453 was appropriately targeted to the endoplasmic reticulum and properly cleaved as it was glycosylated, present in the soluble fraction following Na<sub>2</sub>CO<sub>3</sub> extraction, and accumulated in the medium (Fig. 2B). Compared with equal amounts of NA-WT, the ssNA<sub>32</sub>–453 in the medium inefficiently oxidized to a dimer and possessed only ~2.7% of the

FIGURE 2. Removal of the stalk domain restores secreted NA activity. A, soluble NA constructs with stalk truncations were created by attaching a cleavable ss to residue 32, 39, 47, 57, or 67. B, reducing (RD) immunoblots of the soluble (S) and membrane protein (P) fractions of total vesicles (Tot) prepared from 293T cells transfected with NA-WT-Myc and ssNA<sub>32</sub>–453-Myc that were subjected to Na<sub>2</sub>CO<sub>3</sub> extraction as well as whole cell lysates (WCL) and media. C, cell retained (lysates) and secreted (media) ssNA<sub>32</sub>–453-Myc analyzed in comparison with NA-WT-Myc by nonreducing (NR) and reducing (RD) SDS-PAGE. Note the NA-WT-Myc signal loss upon reduction. The percentage of NA-WT-Myc activity is shown (n = 3) and the oxidized dimers (Ox<sub>di</sub>), monomers (Ox<sub>mon</sub>), and aggregates (Ox<sub>Agg</sub>) are depicted. D, lysates and media from cells transfected with the indicated stalk truncations were analyzed by nonreducing and reducing SDS-PAGE. The activity rate for each construct was normalized for the secreted protein levels in the reducing samples and calculated with respect to ssNA<sub>57</sub>–453-Myc (n = 3). Oxidized (Ox<sub>di</sub>) and SDS-resistant (SDS<sup>R</sup><sub>di</sub>) dimers are indicated.

Contribution of the Transmembrane Domain to NA Assembly
Contribution of the Transmembrane Domain to NA Assembly

NA-WT activity (Fig. 2C and supplemental Fig. 2D). As expected, the ssNA32–453 associated with the cell had even lower activity. Thus, the NA TMD performs a major role in the production of functional NA when the stalk is present but is dispensable for synthesis and trafficking.

Removal of the NA Stalk Domain Restores Activity in the Absence of the TMD—Stalk truncations were examined in the absence of the TMD using ssNA to determine what portion of the stalk contributed to the minimal enzymatic activity of the secreted ssNA32–453 (Fig. 2, A and D). All of the constructs accumulated in their respective medium and resolved on nonreducing SDS-PAGE as intermolecular disulfide-bonded dimers or partially SDS-resistant dimers at ~120 kDa (ssNA57–453 and ssNA67–453 do not possess Cys-49). The small N-terminal stalk deletions provided subtle but not significant increases in the activity of the secreted ssNA39–453 and ssNA47–453 compared with the secreted ssNA32–453. Surprisingly, larger stalk deletions dramatically increased the activity as the secreted ssNA57–453 possessed ~20-fold higher activity than equal amounts of the secreted ssNA32–453 and the secreted stalkless NA (ssNA67–453) possessed ~12-fold higher activity. For the secreted ssNA67–453, this was a dramatic contrast from the nonfunctional NA-TM67–453. Only when six additional residues were removed (ssNA63–453), was the majority of activity and secretion lost. Similar, but much lower activity profiles were also obtained from the cell associated fraction for these constructs (supplemental Fig. 2E). These results indicate that in the absence of the TMD, NA either has lower enzymatic activity when the stalk domain is present or is impaired in its proper assembly.

The NA Signal Anchor Sequence Tetramerizes and Traffics Correctly—How does the TMD contribute to the assembly and activity of NA? To investigate this, a construct was created (NA1–74) where the head domain was removed leaving only the TMD and stalk region. NA1–74 received the two expected N-linked glycans, and similar to NA-WT, some species were Endo-H resistant, and all were PNGase F-sensitive (Fig. 3A). This indicated that NA1–74 targets to the endoplasmic reticulum and likely forms a stable conformation that enables it to pass the endoplasmic reticulum quality control system and traffic through the Golgi (37).

The NA TMD is too small for oligomeric analysis by BN-PAGE, so a cysteine cross-linking approach was used. As a test, Ile-48 and Asn-50 in NA-WT were mutated to Cys. On nonreducing SDS-PAGE, both NA-I48C-WT and NA-N50C-WT resolved as intermolecular disulfide-bonded dimers, trimers, and tetramers at the appropriate molecular weight, and these species collapsed to a single band following reduction with DTT (Fig. 3B). This indicated that tetramerization could be observed by this approach, but it was not a quantitative representation of the proportion of tetrameric NA-WT (see supplemental Fig. 2C).

NA1–74 and NA1–74-I48C were examined similar to NA-WT and NA-I48C-WT, with the exception that PNGase F was utilized to remove the mixed glycosylation species (Fig. 3C compare with supplemental Fig. 3A). Similar to NA-WT, NA1–74 appeared as a monomer and oxidized dimer on nonreducing SDS-PAGE, and NA1–74-I48C possessed the additional trimeric and tetrameric disulfide-bonded species, which demonstrates that the TMD and stalk region tetramerize independently of the head domain (Fig. 3C).

N-terminal Dimeric, Trimeric, and Tetrameric Domains Progressively Restore Secreted NA Activity—In the absence of TMDs with precisely known oligomeric states, we tested how a soluble N-terminal tetramerization domain contributed to NA assembly in comparison with trimerization and dimerization domains. To do this, well defined 32 residue dimeric (Di), trimeric (Tri), and tetrameric (Tet) GCN4 coiled-coil domains (30) were inserted after the signal sequence cleavage site in ssNA32–453 and fused to the N terminus of the stalk (Fig. 4A).

The secreted ssDiNA32–453, ssTriNA32–453, and ssTetNA32–453 all formed oxidized dimers (Fig. 4B). When equal amounts

FIGURE 3. The NA TMD forms a tetramer and traffics through the Golgi. A, NA-WT and NA1–74 transfected cell lysates were deglycosylated with PNGase F and Endo-H, separated by reducing (RD) SDS-PAGE and immunoblotted. The number of N-linked glycans and Endo-H resistant (EHR) forms are indicated. B, immunoblots of NA-WT-Myc, NA-I48C-Myc and NA-N50C-Myc, expressed in 293T cells and separated by nonreducing (NR) and reducing SDS-PAGE. Oxidized dimers (Oxdi), trimers (Oxtri), and tetramers dimers (Oxtet) are designated by arrowheads. C, NA1–74-Myc and NA1–74-I48C-Myc transfected cell lysates were deglycosylated with PNGase F and resolved by nonreducing (−DTT) and reducing (+ DTT) SDS-PAGE.
of secreted ssNA32–453, ssD9NA32–453, ssT14NA32–453, and ssT42 NA32–453 were analyzed, a trend of progressive increases in activity were observed with the secreted ssTetNA32–453 having ~20-fold more activity than ssNA32–453 (Fig. 4B). A similar pattern was also observed in the cell lysates but with lower activity (supplemental Fig. 3B). Upon analysis of these secreted constructs by BN-PAGE, it was evident that ssNA32–453 aggregated and did not form discrete oligomers, explaining its low activity, and that two oligomeric forms were observed for ssD9NA32–453, ssT14NA32–453, and ssT42 NA32–453 (Fig. 4C). Based on the activity profile and the increasing ratio of the larger oligomeric species from ssT9NA32–453 to the ssT12NA32–453 and ssT32NA32–453 samples, it was concluded that the larger oligomeric species likely represented properly assembled NA.

Together, these results suggest that the tetrameric TMD is likely the optimum conformation for proper NA assembly when the stalk domain is present and that the impairment of NA assembly in the absence of the TMD is related to stalk-induced aggregation.

**The NA TMD Oligomerizes in a Heterologous System—**TMD interactions can be quantitatively measured using the GALLEX system in *E. coli* (33). In this system, the strength of the TMD interaction is proportional to the decrease in β-galactosidase activity. This is based on the requirement for two LexA repressors to bind to the LexA operator for β-galactosidase expression to be suppressed (Fig. 5A). First, the NA signal anchor sequence with (NA2–74) and without (NA2–34) the stalk domain were analyzed together with a positive control, the dimeric GpA TMD, and a negative control, the G83I mutant TMD in the GpA TMD that has weak oligomerization (33). Even though less NA2–34 and NA2–74 were synthesized, an ~60% decrease in β-galactosidase activity was observed indicative of strong interactions between the NA TMD (Fig. 5, B and E).

As expected, both NA2–34 and NA2–74 integrated into the *E. coli* membrane (supplemental Fig. 3C). More impressively, NA2–74, which possesses Cys-49 in the stalk, formed an oxidized dimer that resulted in the observation of SDS-resistant trimers and tetramers under nonreducing conditions (Fig. 5C). Thus, oligomerization of the NA TMD is conserved in a heterologous system that lacks several eukaryote-specific lipids.

**The NA Stalk Can Contribute to TMD Oligomerization**—The strength of the NA TMD interaction did not differ when the stalk domain was present. However, the stalk domain could contribute to oligomerization to a lesser extent than the TMD. Thus, residues 7–23 in NA2–74 were exchanged with the 17 residue dimeric GpA TMD or the G83I mutant TMD (Fig. 5A). The GpA–NA24–74 chimera displayed a similar interaction strength as NA2–74, lower than GpA, but the G83I–NA24–74 chimera interacted stronger than the G83I TMD alone (Fig. 5E). Furthermore, under nonreducing conditions, the GpA–NA24–74 and G83I–NA24–74 chimeras formed SDS-resistant tetramers similar to NA2–74. This suggested that the stalk can contribute to oligomerization in the presence of a TMD (Fig. 5D).

**Tethering the Stalk with an Oligomeric TMD Optimizes NA Assembly**—To differentiate between the affect of membrane tethering and TMD oligomerization on the synthesis of enzymatically active NA, NA chimeras containing strong (GpA) and weak (G83I) oligomerizing TMDs were created. Analysis of these constructs in 293T-transfected cell lysates revealed that both chimeras formed oxidized dimers and GpA–NA24–453 possessed ~85% of NA-WT activity, whereas G83I–NA24–453 possessed ~55% of NA-WT activity (Fig. 6A).

On BN-PAGE, GpA–NA24–453 and G83I–NA24–453 produced in 293T cells formed tetrameric oligomers but also a stable higher order oligomer above the soluble 669-kDa marker was observed (Fig. 6B). The higher ratio of the non-native oligomeric structure for the G83I–NA24–453 chimera potentially explains the difference in activity. In summary, these data show that the NA head domain can properly assemble on its own, but when the stalk domain is present, its N terminus must be stabilized to prevent it from aggregating. Thus, the NA assembly process is optimized such that the TMD tethers the stalk to the
membrane in a tetrameric conformation that coordinates with the assembly of the tetrameric head domain.

DISCUSSION

The first type A influenza NA structure was determined using subtype 2 (N2) head domains that were isolated from influenza particles with a protease that cleaved in the stalk and removed the TMD (12, 28). The purified head domains retained 100% of the original activity and this structure, combined with later variants, aided in the design of the NA inhibitors zanamivir and oseltamivir, which are the main drugs against influenza (38, 39). These results likely explain why many previous studies did not investigate the role of the TMD in NA assembly but instead focused on how mutations within the TMD affect trafficking, apical sorting, viral incorporation, particle shape, propagation, and virulence (40–44).

Prior to solving the structure of the NA head domain, it was discovered that the stalk length varies within and across NA subtypes (15, 45). In nature, stalk deletions of up to 25 amino acids have been found, and these are thought to be an adaptive feature as they are linked to host range, cell tropism, and are heterogeneously present during a single outbreak (17, 18, 20–23, 46). In support of this, the stalk is believed to position NA with respect to its receptor as stalk insertions can rescue detrimental deletions that do not affect enzymatic activity but instead prevent elution from erythrocytes (16, 19, 20, 24, 45). Thus, NA must be able to adapt to various insertions and/or deletions without negatively impacting its assembly and function.

Our results demonstrate that the tetrameric NA head domain from subtype 1 (N1) can properly assemble on its own. However, when the stalk region is present, this intermediate domain requires the stabilization of its N terminus to prevent it from inhibiting proper NA assembly. The stabilization of the stalk is achieved by tethering it to the membrane, ideally in a tetrameric state that matches the head domain. Together, the interactions within the head and TMD likely compensate for any structural alterations stalk insertions, deletions, or mutations could cause during NA maturation. On the other hand, when the TMD is attached to the head domain, a minimal stalk length (10 amino acids) is required to prevent the TMD from obstructing the head domain assembly.

Tetramerization of the NA TMD was directly investigated by introducing a cysteine residue into a small TMD construct that enabled endoplasmic reticulum-mediated intermolecular disulfide bond formation. An earlier study investigated oligomerization of the NA TMD using a chimera with transferrin. However, only a slight fraction of transferrin shifted in density, which was suggestive of tetramerization (47).
supports our conclusion that in the presence of a less compatible TMD, a large ectodomain can force the assembly process, but optimal assembly involves coordination between the TMD and the large ectodomain.

Previous work demonstrated that NA rapidly dimerizes within 2.5 min and this can occur co-translationally (25, 27). Additionally, conformation specific antibodies revealed a unique time lag of 5 min between NA oligomerization and when NA reached its native tetrameric conformation (25). These findings, combined with our results, suggest that the TMD likely aids in the assembly process both co-translationally and post-translationally. Co-translationally, the TMD could facilitate the dimerization by driving the initial interaction between a nascent and fully synthesized monomer. Post-translationally, the time lag between NA tetramerization and folding into its native conformation could be explained by the requirement for two dimers to meet and undergo conformational changes in both the head and the TMD. To distinguish between these possibilities the faces or residues that drive the TMD, interactions must first be identified.

There are nine NA subtypes, and all of these are believed to function as a tetramer. This raises the question of whether the contribution of the TMD to NA assembly is conserved, and if so, can an analysis of the different subtypes reveal what mediates the TMD interaction? It is possible that the requirement for the TMD is specific to N1 as a secreted N2 was previously shown to tetramerize and possess activity, but a direct comparison to the wild type version was not made (48). Currently, no NA TMD structures are available to aid in understanding these questions.

It is interesting to speculate what selective advantage oligomerization of the NA TMD provides influenza. The data presented here suggests the strong TMD interactions would increase NA stability and hence activity in different host environments. On the negative side, the increased TMD stability could make the stalk more immunogenic and antibodies to the stalk region could possibly disrupt NA tetramerization and function similar to removing the TMD. If this is true, it would explain the high mutation rate within the stalk domain and the correlation between stalk length and the number of glycosylation sites (about two N-linked glycosylation sites are found for every 10 amino acids above a length of 30), which can mask antibody binding domains (Fig. 6C).

More and more evidence is accumulating that single-spanning TMDs perform multiple functions such as specific lipid binding that can alter protein function, trafficking, or processing (49, 50). This study alone raises several questions regarding whether the NA TMD interaction varies between strains and subtypes, does TMD oligomerization or the strength of the oligomerization change enzymatic or viral properties? Or more generally, how conserved is the principle of matching oligomeric states between the TMDs from type I and type II membrane proteins with that of their ectodomain? By continuing to utilize the significant sequence and biochemical data associated with multifaceted viral proteins, it is likely that more functions attributed to TMDs will become evident.

Acknowledgments—We thank Jan-Willem de Gier and Gunnar von Heijne for critical reading of the manuscript, members of the Center for Biomembrane Research for discussions, Daniel Daley for help with BN-PAGE, and Dirk Schneider for providing the GALLEX system.

REFERENCES

1. Long, M., Betrán, E., Thornton, K., and Wang, W. (2003) The origin of new genes: glimpses from the young and old. Nat. Rev. Genet. 4, 865–875
2. Papavasiliou, F. N., and Schatz, D. G. (2002) Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity. Cell 109, S35–44
3. Domingo, E., and Holland, J. J. (1997) RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol.* 51, 151–178
4. Medina, R. A., and Garcia-Sastre, A. (2011) Influenza A viruses: new research developments. *Nat. Rev. Microbiol.* 9, 590–603
5. Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E., and Andino, R. (2006) Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature 439*, 344–348
6. Laver, W. G., and Valentine, R. C. (1969) Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. *Virology 38*, 105–119
7. Gamblin, S. J., and Skehel, J. J. (2010) Influenza hemagglutinin and neuraminidase membrane glycoproteins. *J. Biol. Chem.* 285, 28403–28409
8. Hirst, G. K. (1942) Adsorption of influenza hemagglutinins and viruses by red blood cells. *J. Exp. Med.* 76, 195–209
9. Gottschalk, A. (1957) Neuraminidase: the specific enzyme of influenza virus and *Vibrio cholerae*. *Biochim. Biophys. Acta 23*, 645–646
10. Webster, R. G., and Laver, W. G. (1967) Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. *J. Immunol. 99*, 49–55
11. Palese, P., and Comps, R. W. (1976) Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoracetyl-neuraminic acid (FANA): mechanism of action. *J. Gen. Virol.* 33, 159–163
12. Varghese, J. N., Laver, W. G., and Colman, P. M. (1983) Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. *Nature 303*, 35–40
13. Russell, R. J., Haire, L. F., Stevens, D. J., Collins, P. J., Lin, Y. P., Blackburn, G. M., Hay, A. J., Gamblin, S. J., and Skehel, J. J. (2006) The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature 443*, 45–49
14. Li, Q., Qi, J., Zhang, W., Vavricka, C. J., Shi, Y., Wei, J., Feng, E., Shen, J., Chen, J., Liu, D., He, J., Yan, J., Liu, H., Jiang, H., Tuzikov, A. B., Bovin, O., Ovcharenko, A. V., Anhlan, D., and Malyshev, N. A. (2009) Structural and functional design of potent sialidase-based inhibitors of influenza virus replication. *J. Virol.* 83, 418–423
15. von Itzstein, M., Wu, W. Y., Kok, G. B., Pegg, M. S., Dyason, J. C., Jin, B., Van Phan, T., Smythe, M. L., White, H. F., and Oliver, S. W. (1993) Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature 363*, 418–423
16. Liu, G., Chen, X., and Kim, C. U. (2000) Discovery and development of GS 4104 (oseltamivir): an orally active influenza neuraminidase inhibitor. *Curr. Med. Chem.* 7, 663–672
17. Bos, T. J., Davis, A. R., and Nayak, D. P. (1984) NH2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. *Proc. Natl. Acad. Sci. U.S.A. 81*, 2327–2331
18. Sivasubramanian, N., and Nayak, D. P. (1987) Mutational analysis of the signal-anchor domain of influenza virus neuraminidase. *Proc. Natl. Acad. Sci. U.S.A. 84*, 1–5
19. Mitnall, L. J., and Nayak, D. P. (1996) The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *J. Virol.* 70, 873–879
20. Sorrell, E. M., Song, H., Pena, L., and Perez, D. R. (2010) A 27-amino-acid variability in the transmembrane domain of neuraminidase is a major contributor to influenza virus pathogenicity. *J. Virol.* 84, 1026–1030
21. Barman, S., Adhikary, L., Chakrabarti, A. K., Berns, C., Kawaoka, Y., and Nayak, D. P. (2005) Role of transmembrane domain and cytoplasmic tail of neuraminidase in influenza virus pathogenicity. *J. Virol.* 79, 6508–6515
22. Barman, S., Berns, C., Kawaoka, Y., and Nayak, D. P. (2004) Role of neuraminidase in influenza virus pathogenicity. *J. Virol.* 78, 5258–5269
23. Blok, J., and Air, G. M. (1982) Block deletions in the neuraminidase genes from some influenza A viruses of the N1 subtype. *Virology 118*, 229–234
24. Sivasubramanian, N., and Nayak, D. P. (1987) Mutational analysis of the signal-anchor domain of influenza virus neuraminidase. *Proc. Natl. Acad. Sci. U.S.A. 84*, 1–5
25. Mitnall, L. J., Castrucci, M. R., Murti, K. G., and Webster, R. G. (1999) The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* 73, 1146–1155
26. Hoffmann, T. W., Munier, S., Soubieux, D., Ledevin, M., Esnault, E., Tourdes, A., Croville, G., Guérin, J. L., Quéré, P., Volmer, P., Naffakh, N., and Marc, D. (2012) Length variations in the NA stalk of an H7N1 influenza virus have opposite effects on viral excretion in chickens and ducks. *J. Virol.* 86, 584–588
27. Zhirnov, O. P., Vorobjeva, I. V., Saphonova, O. A., Poyarkov, S. V., Ovcharenko, A. V., Anhlan, D., and Malyshhev, N. A. (2009) Structural and evolutionary characteristics of HA, NA, NS and M genes of clinical influenza A/H3N2 viruses passed in human and canine cells. *J. Clin. Virol.* 45, 322–333
28. Mitnall, L. J., Matrosovich, M. N., Castrucci, M. R., Tuzikov, A. B., Bovin, N. V., Kobasa, D., and Kawaoka, Y. (2000) Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. *J. Virol.* 74, 6015–6020
29. Saito, T., Taylor, G., and Webster, R. G. (1995) Steps in maturation of influenza A virus neuraminidase. *J. Virol.* 69, 5011–5017
30. Potier, M., Mameli, L., Bévilacqua, M. A., and Leclerc, M. (1999) Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferone-α-N-acetylenuramoyl) substrate. *Anal. Biochem.* 281, 296–297
31. Jejcic, A., Daniels, R., Bovin, O., and Skehel, J. J. (2006) Quasispecies diversity determines pathogenesis through cooperation and virus budding. *J. Virol.* 80, 10575–10584
32. Jejcic, A., Daniels, R., Goobar-Larsson, L., Hebert, D. N., and Vahlne, A. (2009) Small molecule targets Env for endoplasmic reticulum-associated protein degradation and inhibits human immunodeficiency virus type 1 propagation. *J. Virol.* 83, 10075–10084
33. Schneider, D., and Engelman, D. M. (2003) GALLEX, a measurement of heterologous association of transmembrane helices in a biological membrane. *J. Biol. Chem.* 278, 3105–3111
34. Miller, J. H. (1992) A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria, Cold Spring Harbor Laboratory Press, Plainview, NY
deletion in the neuraminidase stalk supports replication of an avian H2N2 influenza A virus in the respiratory tract of chickens. *J. Virol.* **84**, 11831–11840

47. Kundu, A., Jabbar, M. A., and Nayak, D. P. (1991) Cell surface transport, oligomerization, and endocytosis of chimeric type II glycoproteins: role of cytoplasmic and anchor domains. *Mol. Cell Biol.* **11**, 2675–2685

48. Paterson, R. G., and Lamb, R. A. (1990) Conversion of a class II integral membrane protein into a soluble and efficiently secreted protein: multiple intracellular and extracellular oligomeric and conformational forms. *J. Cell Biol.* **110**, 999–1011

49. Contreras, F. X., Ernst, A. M., Haberkant, P., Björkholm, P., Lindahl, E., Gönen, B., Tischer, C., Elofsson, A., von Heijne, G., Thiele, C., Pepperkok, R., Wieland, F., and Brügger, B. (2012) Molecular recognition of a single sphingolipid species by a protein’s transmembrane domain. *Nature* **481**, 525–529

50. Barrett, P. J., Song, Y., Van Horn, W. D., Hustedt, E. J., Schafer, J. M., Hadziselimovic, A., Beel, A. J., and Sanders, C. R. (2012) The amyloid precursor protein has a flexible transmembrane domain and binds cholesterol. *Science* **336**, 1168–1171