Influenza A Virus Hemagglutinin Trimerization Completes
Monomer Folding and Antigenicity

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Abstract

Influenza A virus (IAV) remains an important human pathogen largely due to antigenic drift: the rapid emergence of antibody escape mutants which precludes durable vaccination. The most potent neutralizing antibodies interact with cognate epitopes in the globular ‘head’ domain of hemagglutinin (HA), a homotrimeric glycoprotein. The H1 HA possesses five distinct regions defined by a large number of mouse monoclonal antibodies (mAbs): Ca1, Ca2, Cb, Sa, and Sb. Ca1-Ca2 sites require trimerization to attain full antigenicity, consistent with their locations on opposite sides of the trimer interface. Here, we show that full antigenicity of Cb and Sa sites also requires trimerization, as revealed by immunofluorescence microscopy of IAV-infected cells and biochemically by pulse-chase radiolabelling experiments. Surprisingly, epitope antigenicity acquired by trimerization persists following acid triggering of HA globular domains dissociation, and even after proteolytic release of monomeric heads from acid-treated HA. Thus, the requirement for HA trimerization by trimer-specific mAbs mapping to Ca, Cb, and Sa sites is not dependent upon bridging adjacent monomers in the native HA trimer. Rather, complete antigenicity of HA (and by inference immunogenicity) requires a final folding step that accompanies its trimerization. Once this conformational change occurs, HA trimers themselves would not necessarily be required to induce a highly diverse neutralizing response against epitopes in the globular domain.
Introduction

The influenza A virus (IAV) hemagglutinin (HA) glycoprotein attaches virions to target cells by binding terminal sialic acid residues on cell surface glycans (19, 33). As a prototypical homotrimeric type I integral membrane protein, HA is synthesized in the endoplasmic reticulum (ER) of infected cells and transported through the Golgi complex (GC) to the plasma membrane (PM), where it is incorporated into budding virions. A variable number (depending on the strain) of N-linked oligosaccharides are added co-translationally as HA is extruded into the ER through the translocon and subsequently trimmed and modified extensively during HA transport to the cell surface. In addition, the cytoplasmic COOH-terminus of HA is palmitoylated during its intracellular transport, likely in an early GC compartment (30).

HA folding begins co-translationally, as shown by acquisition of intrachain disulfide bonds and by binding of monoclonal antibodies (mAbs) specific for discontinuous epitopes within HA to nascent chains (18). Initial folding of HA monomers is likely completed shortly after chain termination (within a min or two) whereas HA trimerization occurs with a $t_\frac{1}{2}$ of $\sim$5 min (7, 47). The localization of the later process appears to be strain (and perhaps subtype) specific, with prototype H2 (16) and H3 (6) HAs trimerizing in the ER while the A/Puerto Rico/8/34 (PR8) H1 HA trimerizes in either the ER-Golgi intermediate compartment (ERGIC) or the cis-GC (30, 47).
HA is of great medical importance by virtue of its recognition by neutralizing Abs that provide the bulk of protection afforded by prior IAV infection or vaccination (8). The variability of HA neutralizing epitopes enables IAV immune escape (37). Most neutralizing Abs are directed to epitopes in the globular ‘head’ domain of HA, the location of the sialic acid binding site. Abs neutralize infectivity by sterically blocking HA binding to its receptor and by interfering with conformational alterations required for HA-mediated fusion of viral and cellular membranes (25). Fusion occurs after internalization of virions into endosomes, where the acidic pH triggers HA to expose its fusion peptide.

In classical work, now 30 years old, Gerhard and colleagues used a large panel of mAbs to antigenically define PR8 HA (5, 15). This revealed the presence of five overlapping antigenic sites in the globular domain, two highly variable between closely related epidemic strains (strain specific a (Sa) and Sb), and three sites that demonstrate somewhat more cross-reactivity (Ca1, Ca2, and Cb). The Sa and Sb sites are located at the very tip of the HA, while Ca and Cb lie further down within the HA spike.

With increasing concern regarding the effectiveness of IAV vaccination and the possibility for generating vaccines that induce broadly neutralizing Abs (40), there is a renewed interest in understanding not only HA antigenicity but also HA immunogenicity. Since virtually all neutralizing Abs are conformation specific, a critical issue is the generation of epitopes during the progressive
folding of HA. Biogenesis studies show that HA-specific mAbs are exquisitely sensitive to HA conformational status. The Y8-10C2 mAb (designated Sa11 in original publications) defines a Sa epitope concealed upon HA trimerization (47), making Y8-10C2 a probe for conformational changes, including brief conformational excursions that expose the epitope for Ab binding (45). Conversely, HA trimerization is required to generate the H17-L2 (Ca8) epitope, which is located at the HA trimer interface (47). In IAV PR8-infected cells, Y8-10C2 staining of the secretory compartment is limited to the ER while H17-L2 stains the GC and beyond, consistent with HA trimerization occurring in the GC or during transit of HA from ER exit sites to the GC. The Sb-specific mAb H28-E23 (Sb9) binds both HA monomers and trimers, and stains HA throughout the entire secretory pathway (47).

The distinct pattern of staining of HA monomer vs. trimerization dependent-mAbs makes a very simple screening assay for oligomer discrimination by mAbs specific for the distinct HA antigenic sites. While it is expected that some Ca-specific mAbs, whose escape HA mutants that span the trimer interface, are dependent of HA trimerization, it is possible that epitopes recognized by mAbs specific for other sites also require HA trimerization for their formation.

Here, we identify additional HA trimer-specific mAbs and use them to demonstrate that HA antigenicity, and hence folding, is only fully completed upon HA trimerization.
Materials and Methods

Cells

MDCK cells (American Type Culture Collection; Manassas, VA) were maintained in complete medium (Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX-I (Gibco; Grand Island, NY), supplemented with 4.5 g/L D-glucose, 110 mg/L sodium pyruvate, and 7.5% heat-inactivated fetal bovine serum) at 37°C in a humidified atmosphere of 9% carbon dioxide in air.

Virus and Viral Infections

IAV PR8 was grown in the allantoic cavity of embryonated chicken eggs and stored as infectious allantoic fluid at -80°C. MDCK monolayers were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Gibco) and incubated with IAV PR8 at 10 infectious doses per cell in AIM medium (minimum essential medium (MEM) + GlutaMAX-I (Gibco), supplemented with Earle's salts, 0.1% bovine serum albumin (BSA), and 20 mM Hepes pH 6.6) at 37°C in a humidified atmosphere of 9% carbon dioxide in air. After 1 h, the infection medium was replaced by complete medium and incubation continued for additional 4 h under the same conditions.

Antibodies
Mouse anti-HA mAbs were described in previous publications (5, 15, 41-43, 45, 46). We used mAbs as neat hybridoma culture fluid supernatants in all experiments. Rabbit anti-NA polyclonal Abs were described previously (9). DyLight 488-conjugated donkey anti-mouse IgG (H+L) and DyLight 546-conjugated donkey anti-rabbit IgG (H+L) were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). HRP-conjugated rabbit anti-mouse IgG was purchased from Dako (Carpinteria, CA). HRP-conjugated anti-mouse IgG TrueBlot ULTRA was from eBioscience (San Diego, CA).

**Pulse-Chase Analysis, Immunoprecipitation, and Immunoblotting**

Confluent monolayers of IAV PR8-infected MDCK cells growing in 75 cm² culture flasks (Thermo Scientific Nunc; Rochester, NY) were detached with trypsin (Gibco) and washed twice with DPBS. Cells in suspension were pulse-labeled for 2 min with EasyTag [35S]-Met (PerkinElmer; Waltham, MA) (1 or 0.2 mCi/ml depending whether experiments were performed at 20°C or 37°C, respectively) in Met-free DMEM (Gibco) and then chased in complete medium supplemented with 67 mM L-Met (Sigma-Aldrich; Saint Louis, MO). After each chase time point, cell aliquots were removed and washed once with ice-cold DPBS, supplemented or not with 20 mM N-ethylmaleimide (Calbiochem; Billerica, MA). Cells were pelleted and extracted in ice-cold lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl pH 7.5, 300 mM NaCl and 5 mM EDTA), supplemented with the complete, Mini, EDTA-free protease inhibitor cocktail...
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(Roche Diagnostics, Indianapolis, IN). Equivalent amounts of cell lysates were subjected to immunoprecipitation (IP) as described (3). Immunocollected proteins were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (27) and visualized by exposing dried gels to Carestream Kodak BioMax MR (Sigma-Aldrich) films. Gels were stained with Coomassie brilliant blue R prior to drying to ensure that IgG was equally recovered and loaded in all lanes. Since equal amounts of IgG were added to each sample, this served as a loading control. Immunoblotting studies were performed as described previously (27). Data analysis and quantification were performed using the Image J software (http://rsbweb.nih.gov/ij).

**Immunofluorescence Confocal Microscopy**

MDCK cells (1 x 10^5) growing on 12 mm-microscope cover glasses (Marienfeld GmbH & Co.KG; Lauda-Königshofen, Germany) were infected with IAV PR8 as described above in the presence or absence of 10 μM monensin (Sigma-Aldrich). Cells were washed twice with DPBS supplemented with 0.9 mM CaCl₂ and 0.43 mM MgCl₂ (DPBS-Ca/Mg; Gibco) and then fixed with 3% freshly prepared paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA) in DPBS-Ca/Mg. After permeabilization with 1% Triton X-100, cells were incubated with a mixture of polyclonal anti-NA Abs and the corresponding anti-HA mAb in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.). Cells were then incubated with fluorescent secondary Abs in balanced salt solution (BSS).
supplemented with 0.1% BSA. Inverted cover slips were mounted on microscope slides with Fluoromount G + DAPI (Electron Microscopy Sciences) and analyzed using a TCS SP5 (DMI 6000) confocal microscope system (Leica Microsystems; Deerfield, IL).

**Tryptic Digestion of Recombinant Trimerized HA**

PR8-foldon-His6 recHA (recHA₃) (34) in 1% Triton X-100, 50 mM Tris-HCl pH 7.5, and 100 mM NaCl was incubated at pH 5 (obtained by adding 1 M citric acid pH 1.64) for 15 min at 37°C, returned to neutral pH with 1 M Tris pH 10.91, and exposed to increasing amounts of trypsin TPCK-treated (Worthington; Lakewood, NJ) for additional 15 min at 37°C. Digestion was stopped by adding 1 mM phenylmethanesulfonylfluoride (PMSF) to each sample. Native recHA₃, acid-treated recHA₃, and the monomeric, tryptic fragment derived from recHA1 (sample 7; Fig. 5A) were subjected to IP at high (300 mM) or low (150 mM) concentration of NaCl using specific anti-HA Ca, Cb, Sa, and Sb mAbs.

**Zonal Sedimentation Analysis on Sucrose Gradients**

Trimerized recHA (recHA₃) and monomeric, tryptic recHA1 fragments were analyzed by ultracentrifugation on layered 5-25% (w/v) sucrose gradients (9 ml) with a 2 ml 60% sucrose cushion prepared in 50 mM Tris-HCl pH 7.5 and 100 mM NaCl. Native recHA₃, acid-triggered recHA₃, and tryptic recHA1 were loaded on the top of separate sucrose gradients and centrifuged in a SW41 rotor (Beckman Coulter Inc.; Fullerton, CA) at 35,000 rpm for 16 h at 4°C. Fractions of
0.25 ml were collected from the top of the tube and analyzed by immunoblotting using CM-1, a specific mAb to denatured HA1. The protein standards carbonic anhydrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa) (GE Healthcare Biosciences; Pittsburgh, PA) were loaded at 60 µg/gradient and detected by staining nitrocellulose membranes with Ponceau S (Sigma-Aldrich) before immunoblotting. Standards and recHA proteins on sucrose gradients fractions were quantified by densitometry using the Image J software.

**Surface Plasmon Resonance**

Steady-state equilibrium binding of mAbs to different recHA proteins was monitored using a ProteOn surface plasmon resonance biosensor (Bio-Rad; Hercules, CA) (21, 22). Protein A/G was coupled to GLC sensor chips with amine coupling at 500 resonance units in test flow cells. mAbs in serum-free culture supernatants were captured with protein A/G at 10 µg/ml. 2-fold serial dilution of freshly prepared recHA proteins (starting concentration: 20 µg/ml) were injected at a flow rate of 50 µl/min (contact time: 180 sec) for association, and further disassociation was performed over an interval of 600 sec at a flow rate of 50 µl/min. Responses from the mAb-coated surface were corrected using a mock surface and a separate injection of buffer only. The anti-CCR5 2D7 mAb was included as a negative control. The Bio-Rad ProteOn manager software (version 3.0) was used for data analysis.
Results

Characterizing Nascent HA Monomers and Trimers via SDS-PAGE

As reported previously (47), after 2 min labeling of IAV PR8-infected MDCK cells with [35S]-Met at 37°C, the anti-HA Y8-10C2 and H17-L2 mAbs recovered distinct biochemical species from Triton X-100 lysates as revealed by SDS-PAGE under reducing and non-reducing conditions (Fig. 1, A and B). Under reducing conditions HA migrated more slowly due to complete unfolding of domains maintained by disulfide bonds.

Reduced Y8-10C2-reactive HA migrated with a M_r (65 kDa) expected for glycosylated HA (termed HA) (Fig. 1A). Immediately after pulse-labeling, Y8-10C2 also recovered a ladder of lower M_r species that likely represent incomplete nascent chains that co-translationally fold to create the Y8-10C2 epitope (18). Under non-reducing conditions we observed several minor higher M_r species that may represent host proteins bound post-lysis to HA via disulfide bonds since they were not present after alkylation free Cys residues by adding N-ethylmaleimide (NEM) to the lysis buffer. The addition of NEM also revealed HA with partial intrachain disulfide bonds, termed IT1 and IT2 by Braakman and colleagues (4). Y8-10C2-reactive HA decayed over the chase period due to steric interference of Y8-10C2 access to its epitope coincident with HA trimerization (45, 47) (Fig. 1, A and D).
The same experiment demonstrated HA trimerization by time-dependent increase in recovery of HA by H17-L2 (Fig. 1, B and D). HA exhibited initial slight increase in mobility likely due to trimming of N-linked oligosaccharides in the early GC followed by a more substantial decrease in mobility likely due to additions of extra oligosaccharide chains in the distal GC. Under non-reducing conditions HA trimers migrated as a mixture of monomers, dimers, and trimers. Inclusion of NEM to the extraction buffer prevented the appearance of denaturation-resistant multimers (all samples are boiled in SDS prior to PAGE), indicating that their generation is due to post-lysis disulfide bonds formation between free Cys residues. Since bromelain cleavage of the COOH-terminus of HA prevents the appearance of HA oligomers under non-reducing SDS-PAGE (47), the relevant Cys residues are almost certainly among the three Cys present in the cytoplasmic domain of HA. Using the anti-HA H28-E23 mAb, which recognizes both HA monomers and trimers, to recover HA from detergent lysates gave the expected pattern of HA species in summing the patterns obtained from Y8-10C2 and H17-L2 (Fig. 1, C and D).

To analyze HA folding and trimerization with greater detail we slowed HA biogenesis by performing the pulse-chase protocol at 20°C. This not only increased the amount of incomplete HA chains recovered by Y8-10C2 and H28-E23 but also retarded their resolution into full length HA (Fig. 1, E and G). Completion of HA synthesis required ~10 min under these conditions, as inferred from the maximal amount of HA recovered by Y8-10C2 (Fig. 1H). At this
time, a fairly large fraction of HA had already trimerized, as shown by the kinetics of HA recovery by H17-L2 and H28-E23 (Fig. 1, F-H).

Sequential Immunoprecipitation Clearly Demarcates Y8-10C2- vs. H17-L2-Reactive HA

To test the mutual exclusivity of the Y8-10C2 and H17-L2 mAbs to HA we performed sequential IP, depleting HA species from pulse-chase lysates with one mAb to completion and then analyzing for HA reactive with other mAbs by non-reducing SDS-PAGE. Importantly, we used pulse conditions such that HA monomers and trimers each consisted of ~50% of the total HA pool. Depleting with Y8-10C2 or H17-L2 completely removed each homologous species without significantly affecting recovery of the heterologous species qualitatively or quantitatively (Fig. 2, A, B, and D). Consistent with this result, each mAb depleted its respective species from HA collected with H28-E23, and reduced the total amount of HA recovered by very close to 50% for each treatment (Fig. 2, C and D).

Together, these results clearly show the existence of two distinct populations of HA, monomer vs. oligomer, each uniquely recognized at high affinity by selected mAbs.

Identification of HA Trimerization-Dependent Cb and Sa Epitopes

We revisited the subcellular localization of HA monomers and trimers (47) using confocal microscopy to image mAb binding to fixed and permeabilized cells. We
identified the entire secretory pathway by staining cells with rabbit polyclonal 
Abs raised to a synthetic peptide corresponding to the COOH-terminus of NA.
These Abs recognize all forms of full length NA, including native tetramers and
denatured monomers in immunoblots (9). Immunofluorescence confirmed that
the Y8-10C2 and H17-L2 mAbs exhibit complementary patterns, with Y8-10C2
staining the ER (Fig. 2, E-G) and H17-L2 staining post-ER structures (GC and
PM) (Fig. 2, K-M), while H28-E23 stained the entire secretory pathway, giving
extensive co-localization with NA (Fig. 2, Q-S). We also observed HA trimers on
puncta scattered throughout the cytoplasm that did not overlap with ER-
associated NA (Fig. 2, K-M), which could represent secretory carriers en route to
the cell surface or HA in recycling endosomes.

PM staining interfered with intracellular staining, due to the tenuity of MDCK
cells (Fig. 2, Q-S). We therefore treated cells with the H+/Na+ ionophore
monensin to slow HA transport through the GC and thus reduce its surface
expression (17, 35, 36). Monensin altered the morphology of the GC-containing
NA (Fig. 2, F and I), which failed to stain with Y8-10C2 (Fig. 2, H-J), but stained
intensely with H17-L2 (Fig. 2, N-P) or H28-E23 (Fig. 2, T-V). Monensin
dramatically redistributed all HA trimer-containing structures into perinuclear
clusters of membranous vesicles and tubules (Fig. 2, N-P). As expected from
binding all HA species, H28-E23 staining represented the combined patterns of
Y8-10C2 and H17-L2 and extensively co-localized with anti-NA Abs staining
throughout the secretory pathway (Fig. 2, Q-S and T-V).
We next examined a mAb panel for HA monomer vs. trimer binding by immunofluorescence microscopy, scoring as HA monomer-specific mAbs to those staining the ER only, HA trimerization-dependent mAbs to those that exclusively stained the GC, and HA monomer/trimerization-dependent mAbs to those exhibiting ER/GC specificity (Table 1). This revealed that, with the exception of Sb-specific mAbs, multiple mAbs specific for the Ca, Cb, and Sa antigenic sites of HA surprisingly stained cells in a HA trimer-specific (GC) pattern.

We selected candidate HA trimer-specific mAbs for biochemical analysis, including the HA Ca-specific H17-L10, Cb-specific H35-C10, and Sa-specific H9-A22 mAbs (staining patterns for these mAbs are shown in Fig. 3, A-R). The locations of amino acid substitutions (H3 numbering) that reduce the affinity of these mAbs more than 10-fold are shown in Fig. 4, A, C, and E, respectively (5). We performed pulse-chase experiments to examine the HA species recovered by these mAbs in non-reducing gels using extracts that had been depleted of HA monomers or trimers by Y8-10C2 and H17-L2, respectively. In supporting the microscopy data, each mAb demonstrated clear HA trimerization-dependence, mimicking the properties of H17-L2 described above (Fig. 2B) in recovering HA only after ~5 min chase and from HA monomer- but not trimer-depleted extracts (Fig. 4, B, D, and F). As a control, we also characterized the HA Sb-specific IC5-4F8 mAb, which stained both the ER and GC (Fig. 3, S-X), and, as predicted, recovered both HA monomers and trimers (Fig. 4, G and H).
HA Trimerization-Dependent Epitopes Are Maintained on Trimer-Derived HA Monomers

Notably, all HA trimerization-dependent mAbs identified in the previous section maintain their ability to inhibit hemagglutination mediated by acid triggered virus (44). Since acid triggering loosens the association of the HA globular domains (31, 44), the binding of HA trimer-specific mAbs may not require bridging between adjacent HA monomers. To examine this possibility, we generated monomeric HA ‘tops’ (comprised of the HA globular domain) (29, 32, 38), by exposing recombinant purified trimeric HA (recHA3) (34) to pH 5, returning to neutral pH, and treating with trypsin. Without acid treatment, recHA3 is converted into HA1 (recHA1; Fig. 5A) and HA2 (recHA2; Fig. 5B) chains by trypsin in a dose-dependent manner, as revealed by immunoblotting with mAbs specific for linear determinants in HA1 (CM-1) or HA2 (RA5-22) (39). Acid exposure, however, increases sensitivity of HA1 to further digestion, converting HA1 into a trypsin-resistant form that is the only CM-1-reactive specie present in sample 7 (Tryptic recHA1; Fig. 5A). We confirmed the oligomeric state of native recHA3, acid-triggered recHA3 (HA trimers with separated globular domains) and HA tops (monomeric HA heads) by zonal sedimentation on 5-25% sucrose gradients. As expected, trimerized recHA3 proteins (~200 kDa) sedimented as discrete peaks immediately following fractions containing aldolase (158 kDa) (Fig. 5, C and D). By contrast, monomeric HA tops (~35 kDa) sedimented on intermediate fractions between those
containing carbonic anhydrase (29 kDa) and ovalbumin (43 kDa) (Fig. 5, C and D).

We then incubated native recHA3, acid-treated recHA3 or HA tops with various mAbs, detecting immunoreactive HA species recovered on protein A beads by immunoblotting with the CM-1 mAb. The acid-dependent binding of Y8-10C2 demonstrates that recHA3 homogeneously maintains a tight trimeric structure (Fig. 5E). Notably, each of the HA trimerization-dependent mAbs examined (H17-L2, H17-L10, H35-C10, and H9-A22) demonstrated no loss of binding to acid-triggered HA, even when the interaction occurred in a high salt buffer (300 mM NaCl) (Fig. 5E). Remarkably, three of the four HA trimerization-dependent mAbs examined (H17-L2, H17-L10, and H9-A22) bound the monomeric, tryptic recHA1 fragment (Fig. 5, E and F). In contrast to HA monomer-reactive mAbs, however, the binding of each of these mAbs to the HA tops was reduced at 300 mM salt (Fig. 5E), consistent with a decreased affinity for HA tops relative to native HA trimers.

To further examine the interaction of mAbs with native recHA3, acid-triggered recHA3, and fragmented recHA1, we measured Ab on- \( (k_{on}) \) and off- \( (k_{off}) \) rates by surface plasmon resonance (SPR) (Table 2). We validated the method using Y8-10C2, which as expected (45), demonstrated temperature-dependent binding to native, but not acid-treated recHA3, and bound acid-triggered recHA3 with a 10-fold higher affinity than untreated recHA3. SPR also revealed that acid
treatment of recHA<sub>3</sub> had little effect on the $k_{on}$ and $k_{off}$ of H28-E23 (HA monomer/trimer binding) or the HA trimer-specific H9-A22 mAb. Moreover, acid treatment of recHA<sub>3</sub> increased both the $k_{on}$ and $k_{off}$ for H17-L10 by ~5-fold, while the $k_{off}$ of H35-C10 decreased ~2-fold (Table 2).

With HA tops, all mAbs demonstrated a decrease in the $K_D$. To some extent, this may reflect underestimating the HA top concentration, which is needed to calculate $k_{on}$. This does not account for relative differences in $k_{on}$ between different mAbs, however, nor the complete lack of binding of H35-C10 which confirms the solution assay data in Fig. 5E. A strength of SPR is that $k_{off}$ is independent of protein concentration. For all mAbs, $k_{off}$ are increased relative to acid-treated recHA<sub>3</sub>, except, rather surprisingly, H17-L10, which actually decreases slightly. In any event, the data clearly demonstrate that mAbs that are specific for HA monomer, trimer, or both forms bind to a monomeric form of HA with $K_D$s that are in the physiological range.

Taken together, these data conclusively indicate that HA epitopes that require trimerization can persist on monomers generated from HA trimers. Thus, trimerization is required to complete HA folding, and not exclusively to align contact residues on adjacent HA monomers.

Discussion
We have explored the relationship between HA oligomerization and fine structure using mAbs as structural probes. It is important to note the enormous advantages conferred by using anti-viral mAbs with neutralizing activity to study the general questions about protein antigenicity and structure. Given that viruses exhibit high mutation rates, it is typically simple to obtain a large panel of escape mutants. With advances in nucleic acid sequencing technology, identification of amino acid substitutions on HA that enable immune escape is now fast and cheap. While not all substitutions will represent contact residues for mAbs binding (45), exceptions are likely to be unusual, and in any event, will typically be easy to distinguish by their physical distance from the clustered location of other escape substitutions. In supporting this notion, X-ray crystallography studies on HA escape mutants in conjunction with crystal structures of mAbs Fabs in complex with trimerized and monomeric HA allowed to Skehel, Knossow, and colleagues conclusively demonstrate the remarkable coincidence between the locations where anti-HA mAbs bind and the sites of amino acid substitutions on HA variants (1, 2, 12, 13, 23). Thus, viral antigens like HA, provide a robust, essentially *self-reporting* system for defining epitope structure.

In a previous publication, we had reported that trimeric PR8 HA migrates as a mixture of monomers, dimers, and trimers in denaturing SDS-PAGE under non-reducing conditions (47). Since we had included the alkylating agent iodoacetamide in the extraction buffer, we concluded that the disulfide bonds in...
the COOH-terminus of HA were not artifactually created post-lysis (47). Here, we show that this conclusion is likely erroneous, since replacing iodoacetamide with NEM prevents oligomerization. This is logical, since the Cys residues implicated in cross-linking of the oligomers are present in the COOH-terminus of HA, which should be exposed to the highly reducing environment of the cytosol. We show that, in addition to HA Ca-specific mAbs, a subset of mAbs specific for both Cb and Sa antigenic sites require HA trimerization for binding and fail to bind HA in the ER. This provides further evidence that PR8 HA trimerization occurs only after export from the ER. While this was highly controversial when first proposed, since the ER was thought to be a general site of oligomer assembly (10, 20), there are now examples of other proteins believed to oligomerize in the GC (26, 28).

Despite the fact that the H17-L10 (Ca) epitope spans the HA trimer interface by escape mutant analysis, we found that this mAb binds to monomeric, trypsin-released HA tops, though with diminished affinity. Although it is formally possible that H17-L10 binding to HA tops is based on recruiting two HA monomers to recreate the epitope, this seems unlikely given (1) the presence of only monomeric HA tops species in solution as assayed by zonal sedimentation analysis on sucrose gradients and (2) the inability of H17-L10 to bind to HA monomers in pulse-chase experiments. Rather, it is likely that the H17-L10 epitope is created by conformational changes that accompany trimerization.
Remarkably, and unlike the other mAbs tested, the kinetics of H17-L10 binding to acid-treated recHA₃ and HA tops is highly similar in SPR, suggesting that while mAb binding may be influenced by mutations in adjacent HA monomers, simultaneous interactions among HA monomers are not necessary to achieve maximal binding energy.

Notably, we show that the HA Cb and Sa epitopes can also require HA trimerization. This might have been expected for the Sa antigenic site, since several critical residues contribute to the HA trimer interface. Nevertheless, as with H17-L10, the H9-A22 (Sa) $k_{off}$ is not greatly affected by fragmentation, consistent with achieving maximal binding energy with monomeric HA derived from trimers. The HA trimerization-dependent binding of the Cb-specific H35-C10 mAb, on the other hand, is surprising, as its epitope is located nearly equidistant from the HA trimer interfaces. Indeed, H35-C10 is the only HA trimer-dependent mAb we studied that does not detectably bind to HA tops, suggesting that its epitope is dependent on subtle conformational elements that require maintained oligomerization. Intriguingly, DuBois and colleagues (11) demonstrated that the HA Cb antigenic site is subject to considerable conformational flexibility, consistent with the idea that H35-C10 detects a Cb conformation on the HA tops that is unfavorable to association with this mAb.

The capacity of HA trimerization-dependent mAbs to bind HA trimer-derived monomers explains our previous observation that these mAbs maintain binding
to the HA of acid-triggered virions as measured by hemagglutination inhibition (44). It is important to note that a subset of HA trimerization-dependent mAbs will actually bridge adjacent HA monomers. Although we have yet to clearly identify such mAbs among our panel of anti-IAV PR8 Abs, X-ray crystallography studies have already revealed an example among known mAbs to IAV H3N2 (1).

It will be of great interest to extend these findings to mAbs specific for the stem region of HA that exhibit virus neutralizing activity. Such Abs offer great promise for thwarting HA antigenic drift. Krammer and colleagues (24) recently reported that inclusion of a trimerization domain to recombinant, soluble HAs is necessary to create the epitopes for several representative HA stem-reactive mAbs. The capacity of such mAbs to distinguish HA biosynthetic intermediates has not, however, been reported to date.

The most important conclusion stemming from these findings is that complete HA antigenicity (and presumably immunogenicity) requires subtle conformational effects that accompany HA trimerization, and can largely persist after HA trimers partially (acid treatment) or even completely (proteolytic degradation) dissociate. Thus, although it is clearly established that folding of HA monomers or even isolated globular domains is sufficient to generate a significant fraction of epitopes, we have identified a number of mAbs specific for the HA Ca, Cb, and Sa antigenic sites whose epitopes are created (or completed) only upon HA trimerization. Consequently, judging the degree of folding of HA
in vaccines produced from virus or recombinant means requires a diverse panel
comprising multiple mAbs specific for each HA antigenic site. The good news is
that if these findings extend to contemporary HA strains, dissociation of
trimerized HA in vaccine preparations does not necessarily lead to major losses
in the immunogenicity of the globular head domains.

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   376:92-94.


**Figure Legends and Tables**

**Figure 1.** Biochemical characterization of HA maturation, trafficking, and assembly using specific anti-HA mAbs. (A-C) IAV PR8-infected MDCK cells were labeled with [35S]-Met for 2 min at 37°C, chased for the indicated times at the same temperature, and lysed with an ice-cold extraction buffer supplemented (+NEM) or not (-NEM) with N-ethylmaleimide. Detergent lysates were then precipitated using mAbs specific to HA monomers (Y8-10C2; A), HA trimers (H17-L2; B), or both HA species (H28-E23; C). Immunocollected proteins were boiled in LDS sample buffer supplemented (+DTT) or not (-DTT) with dithiothreitol and analyzed by SDS-PAGE and fluorography. IT1 and IT2: HA folding intermediate species; pH: processed HA; rHA: reduced HA. (D) The amount of immunoprecipitated rHA was quantified by densitometry and expressed as arbitrary units (a.u.). (E-G) MDCK cells infected with IAV PR8 were radiolabeled and chased at 20°C instead of 37°C. Cell lysates made at the end of each chase time point were subjected to IP using the Y8-10C2 (E), H17-L2 (F), or H28-E23 (G) mAbs. (H) The amount of precipitated rHA was quantified as in D.
Figure 2. Specificity of anti-HA mAbs to ER-located HA monomers or trimerized HA lying on the Golgi complex. (A-C) IAV PR8-infected MDCK cells were labeled with [35S]-Met and chased at 37°C as described in Fig. 1, A-C. Cell lysates were depleted twice with an irrelevant mAb to the VSV-N protein (10G-4; no depletion), the HA monomer-specific Y8-10C2 mAb (mHA depleted), or the HA trimer-specific H17-L2 mAb (tHA depleted). After two rounds of depletion, cell extracts were incubated with the Y8-10C2 (A), H17-L2 (B), or H28-E23 (C) mAbs. Immunoprecipitated HA species were analyzed under non-reducing conditions by SDS-PAGE and fluorography. (D) Bar graph representing the percentage of pooled HA-associated radioactivity (from 0 to 20 min) relative to total HA (100%) in non-depleted cell lysates. (E-V) MDCK cells were infected with IAV PR8 in the absence (no treatment) or presence of 10 μM monensin. Cells were fixed, permeabilized, and incubated with the Y8-10C2 (E-J), H17-L2 (K-P), or H28-E23 (Q-V) mAbs (green channel) and rabbit polyclonal Abs to NA (red channel). DNA was labeled using DAPI (blue channel). Stained cells were examined by confocal fluorescence microscopy. Bars: 10 μm. Arrowheads point NA co-localizing with HA monomers on the nuclear envelope (ER) of cells labeled with Y8-10C2 and H28-E23.

Figure 3. Reactivity of various anti-HA mAbs assayed by immunofluorescence microscopy. MDCK cells were infected with IAV PR8 in the absence (no treatment) or presence of 10 μM monensin as in Fig. 2, E-V. HA was labeled on fixed and permeabilized cells using specific mAbs to the HA Ca (H17-L10; A-F),
Cb (H35-C10; G-L), Sa (H9-A22; M-R), or Sb (IC5-4F8; S-X) antigenic sites (green channel). NA was detected using rabbit polyclonal Abs (red channel). DNA was labeled with DAPI (blue channel). Stained cells were examined by confocal fluorescence microscopy. Bars: 10 μm. Arrowheads point NA co-localizing with HA monomers on the nuclear envelope (ER) of cells labeled with IC5–4F8.

Figure 4. Targeting of the Ca, Cb, and Sa antigenic sites by HA trimer-specific mAbs. (A, C, E, and G) PyMOL images of the crystal structure of the IAV PR8 HA trimer (14) (RSCB protein database entry: 1RU7) showing amino acid substitutions (red; H3 numbering scheme) on escape mutants to specific mAbs to the HA Ca (A), Cb (C), Sa (E), and Sb (G) antigenic sites. Each of the subunits within the HA oligomer are displayed in light green, pink, and purple. (B, D, F, and H) Detergent extracts from [35S]-Met-labeled and chased cells were left untreated or depleted for HA monomers or trimers as described in Fig. 2, A-C before to be incubated with specific mAbs to the HA Ca (H17-L10; B), Cb (H35-C10; D), Sa (H9-A22; F), or Sb (IC5-4F8; H) antigenic sites. Immunocollected HA species were visualized by SDS-PAGE under non-reducing conditions followed by fluorography.

Figure 5. HA trimer-specific mAbs to the Ca, Cb, and Sa antigenic sites bind to monomers derived from oligomerized HA. (A and B) Untreated (pH 7.5) or acid-triggered (pH 5) recHA3 were incubated with increasing amounts of trypsin for 15 min at 37°C. Digestion was stopped with 1 mM PMSF. recHA1- (A) or recHA2-derived fragments (B) were then visualized by immunoblotting using...
the anti-HA CM-1 or RA5-22 mAbs, respectively. (C and D) In addition to a cocktail of protein standards, native recHA3, acid-treated recHA3, and the monomeric, tryptic fragments derived from recHA1 (HA tops) (sample 7; A) were fractionated by ultracentrifugation on layered 5-25% sucrose gradients as described in “Materials and Methods”. Sucrose gradients fractions were then analyzed by reducing SDS-PAGE and immunoblotting with the CM-1 mAb (D). The amount of sedimented standards and recHA proteins on each fraction was quantified by densitometry and expressed as arbitrary units (a.u.) (C). (E) The recHA3 pH 7.5, recHA3 pH 5, and monomeric, tryptic recHA1 proteins were subjected to IP at high (300 mM) or low (150 mM) salt using the HA monomer-specific Y8-10C2 mAb, the HA trimer-reactive H17-L2, H17L-10, H35-C10, and H9-A22 mAbs, or the mAbs H28-E23 and IC5-4F8 that recognize both HA species. (F) The amount of immunoprecipitated HA tops was quantified as in Fig. 1D.

Table 1. Immunofluorescence-based screening for anti-HA mAbs specificity

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A panel of anti-HA mAbs was analyzed by immunofluorescence microscopy, scoring ER-only staining as HA monomer-specific mAbs, GC-only staining as HA trimer-reactive mAbs, and ER/GC-staining as HA monomer/trimerization-dependent mAbs.

**Table 2. Kinetic/affinity measurements of mAbs binding to recombinant HA proteins by surface plasmon resonance.**

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SPR was performed by coating chip surfaces with various mAbs followed by injections of 10 µg/ml of native recHA03 (pH 7.5), acid-triggered recHA03 (pH 5), or monomeric, tryptic recHA1 (HA tops) at 15°C. For the Y8-10C2 mAb only, the assay was also done at 4°C, where no binding to native recHA03 was observed. Values represent data from two independent experiments.
Figure 4

A

B

C

D

E

F

G

H
Figure 5

A

recHA\_pH 7.5 | recHA\_pH 5
---|---
1 | 1
2 | 2
3 | 3
4 | 4
5 | 5
6 | 6
7 | 7
8 | 8

IB: α-HA1 (CM-1)

---

IB: recHA, recHA\_A1, Tryptic recHA\_A1

Trypsin (mg/ml) 0.01

B

recHA\_A0, pH 7.5

---

IB: α-HA2 (RA6-22)

Trypsin (mg/ml) 0.01

C

Protein standards (a.u.)

---

29 kDa, 43 kDa, 75 kDa, 158 kDa, 440 kDa

Sucrose gradients fractions

D

Tryptic recHA\_A1

---

recHA\_A, pH 7.5

---

recHA\_A, pH 5

---

Sucrose gradients

E

IP: α-HA (high salt)

---

IP: α-HA (low salt)

---

Sucrose gradients

F

Low salt IP

---

Tryptic recHA\_A1 (a.u.)

---