Influenza Hemagglutinin (HA) Stem Region Mutations That Stabilize or Destabilize the Structure of Multiple HA Subtypes

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ABSTRACT

Influenza A viruses enter host cells through endosomes, where acidification induces irreversible conformational changes of the viral hemagglutinin (HA) that drive the membrane fusion process. The prefusion conformation of the HA is metastable, and the pH of fusion can vary significantly among HA strains and subtypes. Furthermore, an accumulating body of evidence implicates HA stability properties as partial determinants of influenza host range, transmission phenotype, and pathogenic potential. Although previous studies have identified HA mutations that can affect HA stability, these have been limited to a small selection of HA strains and subtypes. Here we report a mutational analysis of HA stability utilizing a panel of expressed HAs representing a broad range of HA subtypes and strains, including avian representatives across the phylogenetic spectrum and several human strains. We focused on two highly conserved residues in the HA stem region: HA2 position 58, located at the membrane distal tip of the short helix of the hairpin loop structure, and HA2 position 112, located in the long helix in proximity to the fusion peptide. We demonstrate that a K58I mutation confers an acid-stable phenotype for nearly all HAs examined, whereas a D112G mutation consistently leads to elevated fusion pH. The results enhance our understanding of HA stability across multiple subtypes and provide an additional tool for risk assessment for circulating strains that may have other hallmarks of human adaptation. Furthermore, the K58I mutants, in particular, may be of interest for potential use in the development of vaccines with improved stability profiles.

IMPORTANCE

The influenza A hemagglutinin glycoprotein (HA) mediates the receptor binding and membrane fusion functions that are essential for virus entry into host cells. While receptor binding has long been recognized for its role in host species specificity and transmission, membrane fusion and associated properties of HA stability have only recently been appreciated as potential determinants. We show here that mutations can be introduced at highly conserved positions to stabilize or destabilize the HA structure of multiple HA subtypes, expanding our knowledge base for this important phenotype. The practical implications of these findings extend to the field of vaccine design, since the HA mutations characterized here could potentially be utilized across a broad spectrum of influenza virus subtypes to improve the stability of vaccine strains or components.

Nearly every year seasonal influenza A viruses contribute to hundreds of thousands of human deaths worldwide and place an extraordinary burden on health care systems and the global economy. Of even greater concern is the unpredictable appearance of antigenically novel influenza A strains in humans, with the potential for causing devastating pandemics. A major obstacle for controlling influenza is the diversity of influenza virus A strains that circulate in the avian species, since this provides a vast genetic pool of viral strains to which the human population is immunologically naive (1). Among viruses isolated from aquatic birds such as ducks and gulls, 16 antigenically distinct hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes have been identified to date, although over the past century only a small subset of these (H1N1, H2N2, and H3N2) have acquired the capacity to cross species barriers and subsequently establish lineages in the human population. However, there have been numerous examples of limited human infection or outbreaks of H5, H6, H7, H9, and H10 subtype viruses over the past several years (2–7). Fortunately, such strains were not transmitted efficiently from human to human, but it will be critical to develop a broader understanding of the mechanisms involved in host adaptation, transmission, and pathogenicity of influenza viruses. These multifactorial traits have been genetically linked to a number of the eight gene segments depending on the viral strain, host system, or

phenotypic readout utilized in any particular study; however, properties of the HA often play a particularly significant role (2, 8–20). The receptor binding characteristics of HA have long been associated with host specificity (21–26), but more recently, a greater appreciation has developed for the role of HA stability in transmission, adaptation, and pathogenicity phenotypes (27–36).

HA stability phenotypes are intricately related to the pH at which the HA mediates endosomal membrane fusion during the virus entry process and, from a structural perspective, stability relates primarily to the postcleavage neutral pH conformation of

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the HA trimer. HA is synthesized as a polypeptide precursor (HA0) that oligomerizes during biogenesis in the endoplasmic reticulum to form homotrimers. Cleavage of HA0 into the disulfide-linked HA1-HA2 subunits is required in order to activate virus infectivity (37–39), since this primes the membrane fusion potential of the glycoprotein. Upon cleavage activation, the newly generated hydrophobic N terminus of the HA2 subunit (i.e., the fusion peptide) is relocated to the interior of the trimer, and this transitions the HA0 structure, which is relatively unresponsive to acidic pH, to a metastable conformation that can subsequently be triggered for membrane fusion activity by acidification of endosomes during virus entry (40, 41). The conformational changes that accompany membrane fusion result in an energetically stable helical rod structure that relocates the fusion peptide from within the trimer interior, making it available for insertion into the endosomal membrane (42).

For natural isolates, HA subtypes can vary with regard to stability phenotypes as assayed by pH and temperature and cover a range of fusion pH and kinetics of inactivation (43, 44). In the laboratory, cleaved HAs can be induced to undergo conformational changes at neutral pH by increasing temperature, and these changes are structurally analogous to those that are triggered by acidification (45–47). Furthermore, a direct correlation is demonstrated between fusion pH and temperature at which specific mutants are triggered (i.e., high pH mutants can be triggered at lower temperature) (45). The differences in fusion pH may reflect the differences in the ecological and host environments in which the distinct subtypes evolve an optimal pH threshold for conformational change that balances the need for stability in the environment with the ability of the virion to uncoat in the endosome. A pH of fusion that is too high can lead to inactivation of the virion prior to receptor-mediated endocytosis by the target cell, and a pH of fusion that is too low could lead to viral degradation before the fusion event can occur.

In addition to the potential role for HA stability in the ecology and transmission of influenza viruses, there could also be implications for viral strains to be utilized as vaccine seed stocks or vaccine components. The issue of stability would be particularly relevant for live attenuated vaccines in regard to both the shelf life of the vaccine and the integrity of the vaccine postvaccination. Incorporating a more stable HA could support vaccine durability during storage and transport. This increased stability would also enable the vaccine to withstand the natural barriers of the nasal cavity, including a reduced pH environment, prior to mediating infection of the nasal epithelial cells (48–50).

In order to identify HA mutations that increase or decrease stability across a range of subtypes, we targeted two highly conserved residues in the stem region for further study, K58 and D112 of the HA2 subunit. These residues have been well characterized for H3 HAs, where K58I and D112G mutations are known to increase or decrease HA stability, respectively, without compromising the structural integrity of the major antigenic regions (51–54). Here we report studies on the stability effects of K58I and D112G mutations introduced into a series of expressed HAs representative of both groups of the phylogenetic spectrum and all five clades. We examined the effects of these mutations by comparing their surface expression, cleavage activation, and fusion characteristics with wild-type (WT) HAs of the same subtype, and we demonstrate that the K58I mutation increases HA stability, and the D112G mutation decreased HA stability, across a broad

range of HA subtypes. Among the factors involved in risk assessment of circulating influenza virus A strains for pandemic potential, HA stability is one property that is poorly defined at present (19), and the identification of structural positions involved in HA stability for multiple subtypes should assist in the focus of this task. In addition, the data reported here provide a foundational backbone for vaccine designs and approaches that might benefit from more stable HA glycoproteins.

MATERIALS AND METHODS

Cells and antibodies. Vero cells (ATCC CCL-81) were used for HA plasmid transfection to analyze surface expression of wild-type and mutant HA proteins. Vero cells were also used as effector cells for transfection of the HA-containing plasmids for the quantitative cell-cell fusion assay, described below, and BSR-T7/5 cells (originally obtained from Karl-Klauss Conzelmann) served as the target cells. BHK-21 cells (ATCC CCL-10) were used for transfections of HA plasmids to analyze fusion via syncytium formation as described below.

The antibodies utilized in the present study were as described previously (27). Briefly, the following antibodies were obtained from BEI Resources: goat anti-H1, goat anti-H2, goat anti-H2^{JAP}, goat anti-H3, goat anti-H4, goat anti-H5, goat anti-H7, goat anti-H8, goat anti-H10, goat anti-H11, goat anti-H1^{PR8}, and goat anti-H5^{VN}. We used a rabbit anti-X31 serum for detection of the Aichi HA.

Plasmids. The wild-type HA expression plasmids have been described previously (27). To introduce the 58I and 112G mutations, the wild-type HAs were PCR amplified from the expression plasmids and cloned into pCR-BLUNT, according to the manufacturer's recommendations. The resultant pCR-BLUNT plasmids were utilized as the templates to introduce each mutation using QuikChange methodology. Sequence-verified mutation-positive plasmids were subsequently utilized as a template for PCR by using primers designed for ligation independent cloning (LIC), described previously (27). Briefly, each mutant HA gene was amplified by PCR using a positive (+)-sense primer with the 5' LIC specific flanking sequence of 5'-TACTTCCAATCCATTGTCACCATG and a negative (–)-sense primer with the 5' LIC-specific flanking sequence of 5'-TTATCCACTTCCATTTCTCA. In addition to the sequences shown, each primer had an additional 12 to 18 nucleotides of gene-specific sequence following either the start or the stop codon. We utilized standard LIC procedures for the generation of each HA plasmid, as described previously (27).

Radioactive labeling and immunoprecipitation. Vero cells were transfected with 1 µg of HA plasmid using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. At 16 to 18 h posttransfection, HA-expressing Vero cells were washed twice with phosphate-buffered saline (PBS) and incubated in minimal essential medium (–Met–Cys) at 37°C for 30 min. Cell proteins were exposed to a [³⁵S]methionine pulse by the addition of 25 µCi of [³⁵S]methionine (Perkin-Elmer) to the starvation medium, followed by incubation for 15 min at 37°C. The cells were then washed with PBS and incubated in complete growth medium supplemented with 20× cold methionine at 37°C for 3 h. After the chase period, the cells were washed once with PBS and treated with either Dulbecco modified Eagle medium (DMEM) alone or DMEM supplemented with 5 µg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin (Sigma)/ml at 37°C for 30 min. Where noted, surface proteins were biotinylated with 2 mg of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS (pH 8.0) at an ambient temperature for 30 min. After biotinylation, the cells were washed with PBS (pH 8.0) supplemented with 50 mM glycine to quench the excess biotin. After a final wash in PBS, the cells were lysed with 0.5 ml of radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]). Cell lysates were centrifuged at 16,000 × g for 10 min to pellet cell debris, and cleared lysates were harvested.

Protein G-Dynabeads (Invitrogen) were conjugated to HA-specific

antibody in PBS plus 0.02% Tween 20 at room temperature for 30 min. The beads were then washed with the PBS–0.02% Tween 20 to remove unbound antibody from the solution. The harvested lysates were incubated with 50 μ l of protein G–Dynabeads for 30 min at room temperature to preclear any nonspecific binding. The precleared lysates were then incubated with protein G–antibody complexes for 1 h at room temperature. Protein G–antibody–antigen complexes were washed three times with PBS, resuspended in 35 μ l of 1 $\times$ SDS buffer, boiled for 5 min, and then centrifuged so that the proteins could be separated from the beads and harvested.

For the precipitation of biotinylated proteins, protein G–antibody–antigen complexes were washed three times with PBS, resuspended in 50 μ l of 50 mM Tris–HCl–0.5% SDS, boiled for 5 min, and centrifuged so that the bead eluate could be harvested. Of the 50 μ l of eluate, 25 μ l was subsequently precipitated with Streptavidin M-280 Dynabeads (Invitrogen) to isolate cell surface-expressed HA, and 25 μ l was reserved to determine the total HA of the sample. Next, 100 μ l of Streptavidin M-280 Dynabeads was washed three times with PBS–0.2% bovine serum albumin (BSA) and resuspended in 975 μ l of streptavidin binding buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA). Then, 25 μ l of eluate was added to the streptavidin Dynabeads–streptavidin binding buffer mixture, followed by incubation at room temperature for 1 h. The unbound HA was then washed from the streptavidin Dynabead–HA complex with three washes of PBS–0.2% BSA. The streptavidin Dynabead–HA complex was resuspended in 35 μ l of 1 $\times$ SDS sample buffer and boiled for 5 min, and the bead eluate was harvested. For gel analysis, the 10 μ l of 3 $\times$ SDS sample buffer was added to the 25 μ l of total HA sample to obtain a final volume of 35 μ l.

For gel analysis, 20 μ l of each sample was resolved by SDS–12% PAGE. The gels were fixed with a 50% methanol–10% acetic acid solution for 30 min at room temperature, and fixed gels were dried under a vacuum for 1 h at 80°C prior to exposure on a phosphorimaging screen. Phosphorimaging screens were scanned on a Typhoon Trio variable-mode imager (GE Healthcare Life Sciences). The intensity of the bands corresponding to HA0, HA1, or HA2 was determined using the ImageQuant software, normalized to the methionine content of the appropriate subunit. For analysis of cell surface-expressed HA and comparison of the wild-type and mutant levels, the total level of HA in each lane was quantified, and the amount of surface-expressed HA was determined by equation (cell surface HA/total HA) $\times$ 100, and then the percent relative to wild-type surface expression was calculated as [(% surface expression mutant HA/% surface expression WT HA) $\times$ 100]. The percent cleavage was determined by using the equation [HA2/(HA2 + HA0)] $\times$ 100, and the percent cleavage relative to the wild type was calculated using the equation [(% cleavage mutant HA/% cleavage of WT) $\times$ 100].

Fusion assays. HA-mediated membrane fusion was examined using a quantitative cell–cell fusion luciferase reporter assay and a qualitative syncytia assay. For the luciferase reporter assay, Vero cells, the effector cells, were transfected with 1 μ g of HA plasmid and 1 μ g of plasmid containing a firefly luciferase gene under the control of a T7 bacteriophage promoter using Lipofectamine and Plus reagent. At 16 to 18 h posttransfection, the HA-expressing cells were washed with PBS, treated with DMEM plus 5 μ g of TPCK–trypsin/ml for 30 min, washed again with PBS, and treated with DMEM plus 20 μ g of trypsin inhibitor/ml for 30 min. The Vero cells were then overlaid with the target BSR–T7/5 cells that constitutively express bacteriophage T7 RNA polymerase. The Vero cells plus the BSR–T7/5 overlay were incubated at 37°C for 1 h to allow the BSR–T7/5 cells to adhere to the Vero cell monolayer. After this incubation, the plates were washed gently with PBS. The cells were exposed to PBS that had been pH adjusted with 100 mM citric acid to the requisite pH and incubated at 37°C for 5 min. At the conclusion of the pH pulse, the pH-adjusted PBS was removed, and complete growth medium was added to neutralize the reaction. The cells were incubated at 37°C for 6 h to allow for fusion of the effector and target cells and to mediate the transfer and expression of the T7 luciferase plasmid for the generation of luciferase protein. The cells

TABLE 1 Origin of HA subtype proteins

Host species and subtype	Strain name
Avian	
H1N1	A/duck/Alberta/35/1976
H2N3	A/duck/Germany/1215/1973
H3N8	A/duck/Ukraine/1/1963
H4N6	A/duck/Czechoslovakia/1/1956
H5N3	A/tern/South Africa/1961
H7N3	A/turkey/England/1963
H8N4	A/turkey/Ontario/6118/1968
H10N7	A/chicken/Germany/N/1949
H11N6	A/duck/England/1/1956
Human	
H1N1	A/Puerto Rico/8/1934
H1N1	A/Georgia/F322551/2012
H2N2	A/Japan/305/1957
H3N2	A/Aichi/2/1968
H5N1	A/VietNam/1204/2004

were washed once with PBS and then lysed with 0.5 ml of reporter lysis buffer (Promega). The cell lysates were frozen for up to 16 h and then, upon thawing, centrifuged at 15,000 $\times$ g at 4°C to remove debris. Then, 150 μ l of the clarified lysate was transferred to a 96-well plate (white, polystyrene; Corning), and the luciferase activity resulting from successful fusion of the target and effector cells was quantified using a Bio-Tek Synergy 2 Luminometer with 50 μ l of luciferase assay substrate (Promega) injected into each well.

For the qualitative syncytium assay, BHK-21 cells were transfected with 1 μ g of plasmid–HA using Lipofectamine and Plus reagent (Invitrogen). At 16 to 18 h posttransfection, HA-expressing cells were washed with PBS and treated with 5 μ g TPCK–trypsin (Sigma)/ μ l in serum-free DMEM for 15 min at 37°C. The trypsin-treated HA-expressing cells were exposed to PBS that had been pH adjusted with 100 mM citric acid, followed by incubation at 37°C for 5 min. At the conclusion of the pH pulse, the pH-adjusted PBS was removed, and the HA-expressing cells were neutralized by the addition of complete growth medium and incubated for 2 h at 37°C to allow syncytia to form. The cells were then stained with the Hema3Stat Pak according to the manufacturer's instructions. Syncytia were visualized and photographed using a Zeiss Axio Observer inverted microscope with an attached digital camera.

RESULTS

Viral strains, mutagenesis, cloning, and expression. For our mutagenesis studies, we utilized cloned HA genes derived from World Health Organization reference strains for our complement of avian HA subtypes, a selection of H1, H2, and H3 subtype HA genes from human isolates, a lab-adapted H1N1 vaccine strain, and a well-characterized H5N1 isolate (Table 1). These strains were chosen in order to sample a wide distribution of host species, dates and geographic locations of virus isolation, and subtypes that span the range of all phylogenetic clades (Fig. 1). The codons for HA2 mutations K58I and D112G were introduced individually into the parental WT HA genes by site-directed mutagenesis, and cloned into a pCAGGS vector for mammalian cell expression under the control of the cytomegalovirus immediate-early promoter. Cells were transfected with these HA expression vectors for all experiments described below addressing HA transport to the plasma membrane, cleavage activation of membrane fusion potential, and the pH at which HA-mediated fusion is activated.

Levels of cell surface expression were determined for WT, K58I, or D112G HAs by surface biotinylation of expressing cells,

FIG 1 (A) The H3 subtype monomer is shown in the central panel with HA1 depicted in blue and HA2 depicted in red. The structural locations of HA2 K58 and HA2 D112 are indicated. In the upper left region of the figure, the head domains corresponding shaded portion of the monomer are shown, with the H3 head domain (blue) superimposed on the H1 (red). The depiction indicates the higher location relative to HA stems for the H1 subtype, and the “twist” in rotational symmetry. The lower left panel shows the HA2 hairpin structures of representatives of four of the five clades superimposed on one another and demonstrates that all are very similar in the long helix domain, but vary in structure for the connecting peptide and top of the short helix (color-coded to match panel B). The bottom right panels correspond to the lower shaded region of the monomer structure and highlight both highly conserved residues and those that segregate in group-specific fashion. HA2 K51, D109, and D112 (top to bottom) are highly conserved, whereas HA2 R/H106, E/Q105, H/T111, and HA1 Y/H17 are conserved within groups. FP indicates the HA2 N-terminal fusion peptide domain, and the spheres denote the HA2 N-terminal glycine residue. (B) This panel shows a phylogenetic tree of the 16 subtypes that circulate in avian species, with the subtypes belonging to group 1 and group 2 indicated and the five clades color-coded to designate those subtypes more closely related by sequence and structure.

followed by immunoprecipitation with specific antisera. **Figure 2** summarizes the results for surface expression of mutant HAs for three or more replicate experiments for each mutant, which we present as the percent surface expression relative to the corresponding wild-type HA. All WT and mutant HAs were found to express on cell surfaces, with an overall range of expression levels comparable to those published previously for mutants HAs (54–56). H5 and H7 subtypes are cleaved by endogenous proteases, so both the cleaved and the uncleaved forms of HA are detectable in the surface expression assay (**Fig. 2B**).

Cleavage activation of membrane fusion potential. The membrane fusion potential of WT HA for all subtypes examined here can be activated by trypsin cleavage of expressed HA0 into HA1 and HA2 (27). To address any possible effects that the 58I or 112G mutations might have on cleavage activation, the degree to which exogenous trypsin processed HA0 into HA1 and HA2 was examined. Pulse-chase experiments were carried out with HA-expressing cells using [35 S]methionine, and monolayers were incubated with trypsin (or media), followed by immunoprecipitation of cell lysates with specific antisera and analysis by SDS-PAGE. As shown in **Fig. 2B** and 3, all mutant HAs were cleaved by trypsin or endogenous proteases, with distinct cleavage products corresponding to the HA1 and HA2 subunits. The cleavage efficiency of the mutant HAs was calculated and expressed as the percent cleavage relative to the wild-type HA (**Fig. 2B** and **Fig. 3**). Although a range of cleavage efficiencies were observed among the mutant HAs, previous studies on expressed HAs of multiple sub-

types suggest that even HAs in the lower end of these ranges are cleaved sufficiently to mediate membrane fusion activity (27).

Membrane fusion pH of mutant HAs by syncytium formation. In order to examine the effects of the 58I and 112G mutations on the fusogenic properties of HA, we used a qualitative syncytium-based fusion assay. BHK cells were transiently transfected with an HA expression plasmid, treated with trypsin (except for H5 and H7 HAs), subsequently exposed to low-pH buffer, and then neutralized. The cells were then incubated for 2 h in standard media (during which syncytia were allowed to form), fixed, stained, and visualized by light microscopy. The pH of fusion was defined as the highest pH value at which extensive syncytia can be detected across multiple fields, and representative photomicrographs are shown in **Fig. 4**. All HAs that were expressed on cell surfaces and cleaved into HA1 and HA2 by trypsin (or endogenous proteases for H5 and H7 HAs) were observed to mediate syncytium formation. In nearly all examples, the K58I mutation led to a lower pH of fusion and the D112G mutation conferred a high pH phenotype relative to the respective WT HA (**Fig. 5** and **Table 2**).

Membrane fusion pH of mutant HAs by quantitative luciferase assay. We also analyzed fusion activity and compared the fusion pH of WT and mutant HAs using a more quantitative luciferase-based assay. This luciferase reporter gene assay provides a numerical measurement that corresponds to fusion, in which fusion is quantified based on the transfer and subsequent expression of a plasmid encoding firefly luciferase under the control of the T7

FIG 2 Surface expression of WT and mutant HAs. Radiolabeled, cell surface proteins were biotinylated and precipitated with an HA-specific antibody and then streptavidin where indicated (SE) and resolved by SDS-PAGE. Lanes are denoted “T” for total HA and “SE” for surface expressed. (A) Gel images for human and avian subtypes are shown. (B) Gel images for H5^{VN}, H5, and H7 are shown. These HAs are expressed in both cleaved and uncleaved forms due to endogenous proteases. (C) Quantitation of the surface expression of mutant HA relative to WT HA. For each WT and mutant HA, the intensity of the bands corresponding to HA0 + streptavidin and HA0 – streptavidin were normalized based on methionine content. The percent surface expression was determined using the following equation (cell surface HA/total HA) × 100, and then the percent relative to wild-type surface expression was calculated as [(% surface expression mutant HA/% surface expression WT HA) × 100]. Error bars represent the standard deviation of three independent experiments.

FIG 3 Analysis of proteolytic cleavage of WT and mutant HAs. Radiolabeled, HA-expressing cells were treated with 5 μ g of TPCK-trypsin/ml, where indicated. Total cellular HA protein was precipitated from the lysate with a HA-specific antibody and resolved by SDS-PAGE. (A) Gel images for human and avian subtypes are shown. (B) Quantitation of trypsin-mediated cleavage of mutant HA relative to WT HA. For each WT and mutant HA, the intensity of the bands corresponding to HA0 and HA2 were normalized based on methionine content. For the H5^{VN}, H5, and H7 subtypes, the cleavage values were determined from the total HA lanes of the surface expression gels shown in Fig. 2B. Percent Cleavage was determined by using the equation $[HA2/(HA2 + HA0)] \times 100\%$, and the percent cleavage relative to wild-type was calculated as $[(\% \text{ cleavage mutant HA} / \% \text{ cleavage of WT}) \times 100]$. Error bars represent the standard deviations of three independent experiments.

FIG 4 Fusion pH as detected by syncytium formation. Photomicrographs of syncytium formation assays for group 1 and group 2, as well as human and avian representative HAs, are shown. HA-expressing BHK cells were treated with TPCK-trypsin (5 µg/ml) and exposed to pH adjusted PBS in 0.1 pH unit increments. Photomicrographs corresponding to the highest pH at which syncytia were observed and then 0.1 pH unit higher are shown.

FIG 5 Syncytium formation assay results for all HA subtypes tested. The substitution H4-K58I did not produce syncytia; the H4-D112G did mediate fusion in the syncytium assay although there was no pH difference between WT and D112G (pH 5.5). H11-K58I (pH 5.8) resulted in a higher pH of fusion than the WT H11 (pH 5.6). H5^{VN}-D112G did mediate fusion; however, the pH of fusion was not higher than that of WT (5.6).

bacteriophage promoter from Vero cells to BSR-T7/5 target cells (27, 55). Assays were carried out using low-pH increments of 0.2 pH units, bracketed based on preliminary results, and data from the syncytium assays. The luminescence associated with each pH value tested was determined, and the pH of fusion was defined as the highest pH value displaying luminescence, after correction by subtracting the value obtained from mock-transfected samples or that of the highest pH well (Fig. 6).

The data from the luciferase assay supports the results from the syncytium assay in all examples (Table 2). Taken together, the two assays demonstrate that the HAs containing the D112G mutation raise the pH of fusion for nearly all subtypes and strains examined, with most displaying fusion activity between 0.2 and 0.5 pH units higher than WT HA, depending on the subtype. This result is consistent with previous observations on D112G mutants and supports a role for the stabilizing effects of hydrogen bonds formed between the highly conserved aspartic acid side chain of

TABLE 2 Fusion pH of wild-type and mutant HAs

Subtype ^a	pH and ΔpH values for wild-type and mutant HAs ^b									
	Wild type		K58I				D112G			
			Luciferase		Syncytia		Luciferase		Syncytia	
	Luciferase	Syncytia	pH	ΔpH	pH	ΔpH	pH	ΔpH	pH	ΔpH
H1	5.6	5.6	5.2	−0.4	5.2	−0.4	5.8	+0.2	5.7	+0.2
H2	5.6	5.5	5.4	−0.2	5.2	−0.3	5.8	+0.2	5.8	+0.3
H3	5.4	5.5	5.2	−0.2	5.1	−0.4	6.0	+0.4	5.8	+0.3
H4	5.4	5.5	ND	ND	ND	ND	5.6	+0.2	5.5	0.0
H5	5.4	5.4	4.8	−0.6	5.0	−0.4	5.6	+0.2	5.6	+0.2
H7	5.2	5.4	4.8	−0.4	4.9	−0.5	5.6	+0.4	5.7	+0.3
H8	5.4	5.4	5.0	−0.4	5.0	−0.4	6.0	+0.6	5.8	+0.4
H10	4.8	5.1	4.4	−0.4	4.4	−0.7	5.4	+0.6	5.4	+0.3
H11	5.6	5.6	5.8	+0.2	5.8	0.2	6.0	+0.4	6.1	+0.5
H1 ^{PR8}	5.2	5.1	5.0	−0.2	5.0	−0.1	5.6	+0.4	5.4	+0.3
H1 ^{GA12}	5.2	5.2	5.0	−0.2	5.0	−0.2	ND	ND	5.7	+0.5
H2 ^{Jap}	5.0	5.2	4.8	−0.2	5.0	−0.2	5.4	+0.4	5.5	+0.3
H3 ^{Aichi}	5.0	5.2	4.8	−0.2	4.7	−0.5	5.4	+0.4	5.5	+0.3
H5 ^{VN}	5.8	5.6	5.2	−0.6	5.2	−0.4	5.8	0.0	5.6	0.0

^a HA designations (from strains in Table 1): PR8, A/Puerto Rico/8/1934; GA12, A/Georgia/F322551/2012; Jap, A/Japan/305/1957; Aichi, A/Aichi/2/1968; VN, A/VietNam/1204/2004.

^b ND, no data available. Changes in pH are indicated in boldface.

D112 and the HA fusion peptide (51, 57). One exception involved the H5^{VN} isolate HA, for which the D112G mutation appeared to have no effect on the pH of fusion. The H1^{GA} isolate HA containing the D112G mutation demonstrated an increase of the fusion pH of 0.5 pH units in the syncytium assay but did not mediate fusion in the luciferase assay. Also, the H4 subtype HA demonstrated only a moderate increase in fusion pH of 0.2 based on the luciferase assay, and no difference to WT HA was observed by syncytium formation.

Conversely, the K58I mutation lowered the pH of fusion by 0.2 to 0.5 pH units for all of the HA subtypes examined with the exception of H11. The H11 subtype is unique among the HA subtypes, since it is the only one encoding an arginine rather than a lysine at the otherwise-invariant HA2 position 58. For the H11 subtype, the pH of the wild-type HA was measured to be 5.6, while the pH of the H11-R58I HA was measured to be 5.8. Fusion was undetectable for the H4-K58I mutation in both the syncytium- and the luciferase-based assay.

DISCUSSION

The phenotypic characteristics of the influenza A virus HA glycoprotein play an important role in the process of virus adaptation to varied hosts and ecological niches, and the capacity to modify the stability of HA constitutes one mechanism for adaptation to dynamic environmental pressures. As a general concept, viral envelope components and capsids must evolve a degree of stability sufficient to protect their genomes as they transmit between hosts, yet maintain the capacity to uncoat in response to specific stimuli when appropriate cells at sites of infection are encountered in a new host. For influenza A viruses, the HA-mediated endosomal uncoating process is generally triggered at a pH between 5.0 and 6.0, depending on the viral strain, whereupon the metastable HA structure undergoes irreversible transitions to a highly stable conformation to induce fusion of the viral and endosomal membranes. However, the irreversible HA conformational changes associated with fusion can also be induced by environmental factors

such as temperature, resulting in the inactivation of virus infectivity. The broad ecological distribution of influenza A viruses, and the diversity of avian and animal species that act as hosts, require that HA maintain an appropriate balance with respect to stability properties and be capable of modulating these properties to adapt to different hosts, modes of transmission, or environmental niches.

H5N1 viruses have received particular attention over the past several years, and evidence is accumulating to demonstrate that HA stability can influence replication and pathogenicity characteristics within particular hosts, as well as adaptation and transmission phenotypes. An optimal range of fusion pH can be demonstrated as a determinant of adaptation and host specificity with avian viruses generally exhibiting a higher activation pH than mammalian strains, presumably corresponding to differences in the sites of infection within these host species. Studies with an avian H5N1 in chickens and ducks reveals an optimal pH range of 5.6 to 6.0 and an increase in pathogenicity corresponding to an increase in the pH of fusion (30, 31), whereas an avian H5N1 studied in mice exhibits an optimal pH of fusion below 5.6, suggesting adaptation of the avian strain to the mammalian host (58). HA stability and fusion pH were also implicated as potential determinants for aerosol transmission of H5N1 strains in the ferret model (28, 29, 32).

It would appear likely that observations made with H5 viruses regarding the biological impact of HA stability properties will translate to other subtypes as well. Sequence alignment and structural characterization of the HAs from the 16 influenza virus A subtypes (excluding the H17 and H18 bat viruses) support the idea that evolutionary pressure operates at the level HA stability and/or membrane fusion properties. The HA subtypes can be segregated into five clades that assemble into two groups (group 1 and group 2; Fig. 1), and while structural comparison of neutral pH cleaved HAs demonstrates a general degree of similarity, structural distinctions are revealed in regions of the molecule that are

FIG 6 Fusion pH as detected by luciferase reporter gene assay. HA-expressing Vero cells transfected with T7-luciferase plasmid were treated with TPCK-trypsin (5 μ g/ml), followed by soybean trypsin inhibitor (20 μ g/ml), and then overlaid with BSR-T7/5 target cells that constitutively express T7 RNA polymerase. The mixed cell population was exposed to pH-adjusted PBS in 0.2 pH unit increments and incubated for 6 h at 37°C to allow for cell-to-cell fusion and content mixing, leading to the expression of firefly luciferase to occur. Luminescence was measured as an indicator of membrane fusion in cell lysates. The graphs show the background-adjusted luminescence. Error bars represent the standard deviations of triplicate experiments. (A) Avian origin HA subtypes. (B) Human origin HA subtypes. No fusion was detected for the H1^{GA}-D112G HA.

rearranged during the fusion process. One difference involves the extended polypeptide chain that links the long and short helices of HA2, which rearranges during fusion to become part of the post-fusion helical rod structure (42). Compared to group 2 HAs, group 1 HAs contain an extra half turn of the short helix that orients the linking peptide into closer proximity to the long helix, and as the chain traces toward the top of the long helix it forms a “taller” hairpin loop. As a result, the membrane distal head domains of group 1 HAs are positioned ~ 4 Å “higher” on the stem domains relative to group 2 HAs. The head domains, which dimerize during fusion, also vary among the subtypes by up to 35° with respect to rotational symmetry along the 3-fold axes (59, 60). In addition, group-specific differences are observed in a cluster of residues in close proximity to the fusion peptide in the cleaved, neutral pH structures, a region that is potentially involved in initial triggering of the acid-induced conformational changes, as fusion peptide residues are expelled from the trimer interior prior to being directed toward the target membrane (Fig. 1).

The structural locations of HA mutations that influence HA stability have been most extensively studied for the HA of H3 subtype viruses, and can map to positions throughout the HA trimer but concentrate at the interfaces of domains that relocate during fusion (57, 61, 62). The locations of such mutations are often coincident with the residues and domains defined as variable by the structural comparison of HA subtypes discussed above, but there are few studies that have compared the stability phenotypes of mutant HAs across subtypes. In this report, we focused on two such mutants at positions that are highly conserved among influenza A HAs. HA2 residue 58, a lysine in all HA subtypes with the exception of H11 (R58), resides in the region of the membrane distal end of the small α -helix, where it meets the extended polypeptide linked to the long helix (Fig. 1). As noted above, the small helix of group 1 HAs is extended by a half turn relative to group 2 HAs, and in group 1 structures residue 58 lies within the helix, and the lysine side chain orients toward the trimer interior and interacts with the glutamic acid side chain of conserved residue 97 in the long helix. In group 1 HAs the short helix contains a serine or glutamine at residue 54, whereas group 2 HAs contain a group-specific arginine at position 54, which interacts with the glutamic acid at position 97 of the long helix. As a consequence, the K58 side chain of group 2 HAs is oriented into solution and the helix is shorter by half a turn. With regard to HA2 residue 112, aspartic acid at this position is completely conserved among all HA subtypes. It is located on the long α helix of HA2 and, in the prefusion conformation of HA, is buried within the trimer interior. Here it forms several H-bond contacts with residues 3, 4, 5, and 6 of the fusion peptide to stabilize the prefusion conformation. When glycine is substituted for aspartic acid, a water molecule occupies the space where the side chain resides in the WT HA, and a weaker network of H-bonds with the fusion peptide is probably responsible for the less stable phenotype (51). As the HA2 58 region differs in group 1 and group 2 HAs, and the region encompassing the HA2 residue 112 contains a number of group-specific ionizable residues that have the potential to influence HA stability (Fig. 1), the phenotypes of mutant HAs at these positions were not entirely predictable.

The data presented here show that all mutant HAs were capable of proper folding and transport to cell surfaces. For all surface-expressed HAs, trypsin or endogenous proteases were able to activate fusion potential by appropriate cleavage into HA1 and HA2,

and all cleaved, surface-expressed HAs demonstrated acid-induced membrane fusion activity. For all of the D112G mutants except the H5 HA of A/Vietnam/1204/04 virus, the mutation caused an elevated pH of fusion relative to the WT HA. This is consistent with results of Russell and coworkers, who observed no change in fusion pH for this mutant HA even though conformational changes could be detected at higher pH than fusion activity (55). It should be noted that the D112G mutant of H5 HA of A/Tern/South Africa/61 virus (H5N3) showed a higher fusion pH than its parental WT, similar to all other D112G mutants examined here. For the HAs with the K58I mutation, all that were expressed on cell surfaces displayed an acid-stable phenotype with the exception of the H11 subtype HA, which is the only HA subtype containing arginine rather than lysine at HA2 position 58 in the WT virus (A/Duck/England/56). This H11 HA is also unusual in that it has glutamine rather than glutamic acid at position 97, possibly altering the interactions between the long and short helices of HA2. However, other than the H11 example, the consistent acid-stable phenotype of K58I HAs across the phylogenetic spectrum is interesting, since group 1 and group 2 HAs deviate in structure in the region of HA2 58. This is also the region where the group-specific antiviral compound TBHQ binds, and its mode of action has been proposed to involve fusion inhibition due to the stabilization of neutral pH HA during acidification (63, 64). The direct interpretation of the stabilizing effects of the K58I mutations will require structural characterization of such HAs at high resolution. Generally, the stabilizing K58I HAs were expressed at higher levels on cell surfaces, whereas the D112G HAs more frequently displayed reduced cell surface expression. Overall, the results reported here are consistent with the limited data on the equivalent mutations in other subtypes (52, 55). The accumulation of data on stability mutants across HA subtypes may provide useful information for identifying viruses with potential for host range adaptation or other biological properties such as transmission and pathogenicity.

The mutant HAs containing K58I, with increased stability properties, could also prove useful as components of influenza vaccines with greater shelf life or as reagents utilized for vaccine assessment. Stability is an issue of universal concern, both for the shelf life of a new vaccine and for the stability of the vaccine particle within the vaccinated host. For influenza viruses, the problem is even more acute as the viral vaccine strains are frequently changed to reflect the circulating strains and subtypes, and the necessity of a quick turnaround prevents extensive temporal studies on stability and shelf life of the vaccine. Therefore, replication characteristics and inherent HA stability, as well as antigenic properties, may be factors to consider for vaccine candidates (50, 65). Recent experiences with live attenuated vaccine strains of H5N1 viruses, as well as H1N1 pandemic strains, highlight the potential problems associated with vaccine stability and potential solutions that might involve the use of HAs with greater stability to pH and temperature dynamics. For example, inefficient replication properties and immunogenicity of live attenuated candidate H5N1 vaccines (66) were improved by the introduction of the K58I acid-stable HA that we address in the current studies (67). Similarly, live attenuated vaccine candidates of pandemic H1N1 strains were less immunogenic when 2009 strains from early in the outbreak were utilized compared to subsequently circulating strains. The critical genetic factor was identified as an HA stalk region HA2 E47K mutation that increased the HA stability of

the stains that circulated in the years that followed the initial outbreak (68). An advantage of stabilizing vaccine candidates using HA stalk mutations is that they would be unlikely to alter the major antigenic sites located on membrane distal HA head domains. Indeed, for the H3 subtype HAs, the K58I mutant and other stalk region mutations have been well characterized for recognition by a wide panel of conformation-specific monoclonal antibodies that target several of the antigenic regions of HA; in nearly all cases, the mutations have no effect on the antigenic properties of mutant HAs (52–54). Acid-stable HAs could also prove useful for improving inactivated vaccines and those based on expressed recombinant proteins, as these would be less susceptible to HA degradation (69, 70), and the expression and purification of bromelain-released HA ectodomains can prove problematic for HAs with elevated fusion pH phenotypes (53, 71). Therefore, incorporation of stabilizing mutations such as K58I might prove useful to improve yield or shelf life of HAs whether such reagents were to be utilized for vaccine purposes, for generating analytical sera, or assessing immune responses to new vaccine candidates and is broadly applicable to a wide range of subtypes across the phylogenetic spectrum of influenza A viruses.

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REFERENCES

- Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev* 56:152–179.
- Herfst S, Imai M, Kawaoka Y, Fouchier RA. 2014. Avian influenza virus transmission to mammals. *Curr Top Microbiol Immunol* 385:137–155. http://dx.doi.org/10.1007/82_2014_387.
- de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. 1997. A pandemic warning? *Nature* 389:554. <http://dx.doi.org/10.1038/39218>.
- Hien TT, de Jong M, Farrar J. 2004. Avian influenza: a challenge to global health care structures. *N Engl J Med* 351:2363–2365. <http://dx.doi.org/10.1056/NEJMp048267>.
- Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. *N Engl J Med* 368:1888–1897. <http://dx.doi.org/10.1056/NEJMoa1304459>.
- Claas EC, Osterhaus AD, van Beek R, de Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351:472–477. [http://dx.doi.org/10.1016/S0140-6736\(97\)11212-0](http://dx.doi.org/10.1016/S0140-6736(97)11212-0).
- Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, Lochindarat S, Nguyen TK, Nguyen TH, Tran TH, Nicoll A, Touch S, Yuen KY, Writing Committee of the World Health Organization Consultation on Human Influenza. 2005. Avian influenza A (H5N1) infection in humans. *N Engl J Med* 353:1374–1385. <http://dx.doi.org/10.1056/NEJMr052211>.
- Almond JW. 1977. A single gene determines the host range of influenza virus. *Nature* 270:617–618. <http://dx.doi.org/10.1038/270617a0>.
- Oxford JS, McGeoch DJ, Schild GC, Beare AS. 1978. Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. *Nature* 273:778–779. <http://dx.doi.org/10.1038/273778a0>.
- Scholtissek C, Burger H, Kistner O, Shortridge KF. 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology* 147:287–294. [http://dx.doi.org/10.1016/0042-6822\(85\)90131-X](http://dx.doi.org/10.1016/0042-6822(85)90131-X).
- Scholtissek C, Koennecke I, Rott R. 1978. Host range recombinants of fowl plague (influenza A) virus. *Virology* 91:79–85. [http://dx.doi.org/10.1016/0042-6822\(78\)90356-2](http://dx.doi.org/10.1016/0042-6822(78)90356-2).
- Scholtissek C, Murphy BR. 1978. Host range mutants of an influenza A virus. *Arch Virol* 58:323–333. <http://dx.doi.org/10.1007/BF01317824>.
- Snyder MH, Buckler-White AJ, London WT, Tierney EL, Murphy BR. 1987. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. *J Virol* 61:2857–2863.
- Snyder MH, Clements ML, De Borde D, Maassab HF, Murphy BR. 1985. Attenuation of wild-type human influenza A virus by acquisition of the PA polymerase and matrix protein genes of influenza A/Ann Arbor/6/60 cold-adapted donor virus. *J Clin Microbiol* 22:719–725.
- Snyder MH, Betts RF, DeBorde D, Tierney EL, Clements ML, Herrington D, Sears SD, Dolin R, Maassab HF, Murphy BR. 1988. Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. *J Virol* 62:488–495.
- Chanock RM, Murphy BR, Collins PL, Coelingh KV, Olmsted RA, Snyder MH, Spriggs MK, Prince GA, Moss B, Flores J, et al. 1988. Live viral vaccines for respiratory and enteric tract diseases. *Vaccine* 6:129–133. [http://dx.doi.org/10.1016/S0264-410X\(88\)80014-8](http://dx.doi.org/10.1016/S0264-410X(88)80014-8).
- de Wit E, Kawaoka Y, de Jong MD, Fouchier RA. 2008. Pathogenicity of highly pathogenic avian influenza virus in mammals. *Vaccine* 26(Suppl 4):D54–D58. <http://dx.doi.org/10.1016/j.vaccine.2008.07.072>.
- Steinhauer DA, Skehel JJ. 2002. Genetics of influenza viruses. *Annu Rev Genet* 36:305–332. <http://dx.doi.org/10.1146/annurev.genet.36.052402.152757>.
- Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, Herfst S, van Boheemen S, Linster M, Schrauwen EJ, Katelnick L, Mosterin A, Kuiken T, Maher E, Neumann G, Osterhaus AD, Kawaoka Y, Fouchier RA, Smith DJ. 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* 336:1541–1547. <http://dx.doi.org/10.1126/science.1222526>.
- Brown EG, Liu H, Kit LC, Baird S, Nesrallah M. 2001. Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. *Proc Natl Acad Sci U S A* 98:6883–6888. <http://dx.doi.org/10.1073/pnas.111165798>.
- de Graaf M, Fouchier RA. 2014. Role of receptor binding specificity in influenza A virus transmission and pathogenesis. *EMBO J* 33:823–841. <http://dx.doi.org/10.1002/emboj.201387442>.
- Wilks S, de Graaf M, Smith DJ, Burke DF. 2012. A review of influenza haemagglutinin receptor binding as it relates to pandemic properties. *Vaccine* 30:4369–4376. <http://dx.doi.org/10.1016/j.vaccine.2012.02.076>.
- Mair CM, Ludwig K, Herrmann A, Sieben C. 2014. Receptor binding and pH stability: how influenza A virus hemagglutinin affects host-specific virus infection. *Biochim Biophys Acta* 1838:1153–1168. <http://dx.doi.org/10.1016/j.bbame.2013.10.004>.
- Xiong X, McCauley JW, Steinhauer DA. 2014. Receptor binding properties of the influenza virus hemagglutinin as a determinant of host range. *Curr Top Microbiol Immunol* 385:63–91. http://dx.doi.org/10.1007/82_2014_423.
- Connor RJ, Kawaoka Y, Webster RG, Paulson JC. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 205:17–23. <http://dx.doi.org/10.1006/viro.1994.1615>.
- Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y. 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol* 74:8502–8512. <http://dx.doi.org/10.1128/JVI.74.18.8502-8512.2000>.
- Galloway SE, Reed ML, Russell CJ, Steinhauer DA. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS Pathog* 9:e1003151. <http://dx.doi.org/10.1371/journal.ppat.1003151>.
- Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmis-

- sion to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 486:420–428. <http://dx.doi.org/10.1038/nature10831>.
29. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336:1534–1541. <http://dx.doi.org/10.1126/science.1213362>.
 30. Reed ML, Bridges OA, Seiler P, Kim JK, Yen HL, Salomon R, Govorkova EA, Webster RG, Russell CJ. 2010. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks. *J Virol* 84:1527–1535. <http://dx.doi.org/10.1128/JVI.02069-09>.
 31. DuBois RM, Zaraket H, Reddivari M, Heath RJ, White SW, Russell CJ. 2011. Acid stability of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity. *PLoS Pathog* 7:e1002398. <http://dx.doi.org/10.1371/journal.ppat.1002398>.
 32. Shelton H, Roberts KL, Molesti E, Temperton N, Barclay WS. 2013. Mutations in haemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. *The J Gen Virol* 94:1220–1229. <http://dx.doi.org/10.1099/vir.0.050526-0>.
 33. Collins PJ, Vachieri SG, Haire LF, Ogorodowicz RW, Martin SR, Walker PA, Xiong X, Gamblin SJ, Skehel JJ. 2014. Recent evolution of equine influenza and the origin of canine influenza. *Proc Natl Acad Sci U S A* 111:11175–11180. <http://dx.doi.org/10.1073/pnas.1406606111>.
 34. Koerner I, Matrosovich MN, Haller O, Staeheli P, Kochs G. 2012. Altered receptor specificity and fusion activity of the haemagglutinin contribute to high virulence of a mouse-adapted influenza A virus. *The J Gen Virol* 93:970–979. <http://dx.doi.org/10.1099/vir.0.035782-0>.
 35. Bottcher-Friebertshauser E, Garten W, Matrosovich M, Klenk HD. 2014. The hemagglutinin: a determinant of pathogenicity. *Curr Top Microbiol Immunol* 385:3–34. http://dx.doi.org/10.1007/82_2014_384.
 36. Belser JA, Gustin KM, Pearce MB, Maines TR, Zeng H, Pappas C, Sun X, Carney PJ, Villanueva JM, Stevens J, Katz JM, Tumpey TM. 2013. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. *Nature* 501:556–559. <http://dx.doi.org/10.1038/nature12391>.
 37. Klenk HD, Rott R, Orlich M, Blodorn J. 1975. Activation of influenza A viruses by trypsin treatment. *Virology* 68:426–439. [http://dx.doi.org/10.1016/0042-6822\(75\)90284-6](http://dx.doi.org/10.1016/0042-6822(75)90284-6).
 38. Lazarowitz SG, Choppin PW. 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. *Virology* 68:440–454. [http://dx.doi.org/10.1016/0042-6822\(75\)90285-8](http://dx.doi.org/10.1016/0042-6822(75)90285-8).
 39. Appleyard G, Maber HB. 1974. Plaque formation by influenza viruses in the presence of trypsin. *J Gen Virol* 25:351–357. <http://dx.doi.org/10.1099/0022-1317-25-3-351>.
 40. Wilson IA, Skehel JJ, Wiley DC. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289:366–373. <http://dx.doi.org/10.1038/289366a0>.
 41. Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC. 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* 95:409–417. [http://dx.doi.org/10.1016/S0092-8674\(00\)81771-7](http://dx.doi.org/10.1016/S0092-8674(00)81771-7).
 42. Bullough PA, Hughson FM, Skehel JJ, Wiley DC. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371:37–43. <http://dx.doi.org/10.1038/371037a0>.
 43. Scholtissek C. 1985. Stability of infectious influenza A viruses to treatment at low pH and heating. *Arch Virol* 85:1–11. <http://dx.doi.org/10.1007/BF01317001>.
 44. Scholtissek C. 1985. Stability of infectious influenza A viruses at low pH and at elevated temperature. *Vaccine* 3:215–218. [http://dx.doi.org/10.1016/0264-410X\(85\)90109-4](http://dx.doi.org/10.1016/0264-410X(85)90109-4).
 45. Ruigrok RW, Martin SR, Wharton SA, Skehel JJ, Bayley PM, Wiley DC. 1986. Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. *Virology* 155:484–497. [http://dx.doi.org/10.1016/0042-6822\(86\)90210-2](http://dx.doi.org/10.1016/0042-6822(86)90210-2).
 46. Carr CM, Chaudhry C, Kim PS. 1997. Influenza hemagglutinin is spring-loaded by a metastable native conformation. *Proc Natl Acad Sci U S A* 94:14306–14313. <http://dx.doi.org/10.1073/pnas.94.26.14306>.
 47. Haywood AM, Boyer BP. 1986. Time and temperature dependence of influenza virus membrane fusion at neutral pH. *J Gen Virol* 67(Pt 12):2813–2817.
 48. Fischer H, Widdicombe JH. 2006. Mechanisms of acid and base secretion by the airway epithelium. *J Membr Biol* 211:139–150. <http://dx.doi.org/10.1007/s00232-006-0861-0>.
 49. Fischer H, Widdicombe JH, Illek B. 2002. Acid secretion and proton conductance in human airway epithelium. *Am J Physiol Cell Physiol* 282:C736–C743. <http://dx.doi.org/10.1152/ajpcell.00369.2001>.
 50. O'Donnell CD, Vogel L, Matsuoka Y, Jin H, Subbarao K. 2014. The matrix gene segment destabilizes the Acid and thermal stability of the hemagglutinin of pandemic live attenuated influenza virus vaccines. *J Virol* 88:12374–12384. <http://dx.doi.org/10.1128/JVI.01107-14>.
 51. Weis WI, Cusack SC, Brown JH, Daniels RS, Skehel JJ, Wiley DC. 1990. The structure of a membrane fusion mutant of the influenza virus haemagglutinin. *EMBO J* 9:17–24.
 52. Steinhauer DA, Wharton SA, Skehel JJ, Wiley DC, Hay AJ. 1991. Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. *Proc Natl Acad Sci U S A* 88:11525–11529. <http://dx.doi.org/10.1073/pnas.88.24.11525>.
 53. Steinhauer DA, Martin J, Lin YP, Wharton SA, Oldstone MB, Skehel JJ, Wiley DC. 1996. Studies using double mutants of the conformational transitions in influenza hemagglutinin required for its membrane fusion activity. *Proc Natl Acad Sci U S A* 93:12873–12878. <http://dx.doi.org/10.1073/pnas.93.23.12873>.
 54. Thoennes S, Li ZN, Lee BJ, Langley WA, Skehel JJ, Russell RJ, Steinhauer DA. 2008. Analysis of residues near the fusion peptide in the influenza hemagglutinin structure for roles in triggering membrane fusion. *Virology* 370:403–414. <http://dx.doi.org/10.1016/j.virol.2007.08.035>.
 55. Reed ML, Yen HL, DuBois RM, Bridges OA, Salomon R, Webster RG, Russell CJ. 2009. Amino acid residues in the fusion peptide pocket regulate the pH of activation of the H5N1 influenza virus hemagglutinin protein. *J Virol* 83:3568–3580. <http://dx.doi.org/10.1128/JVI.02238-08>.
 56. Cross KJ, Wharton SA, Skehel JJ, Wiley DC, Steinhauer DA. 2001. Studies on influenza haemagglutinin fusion peptide mutants generated by reverse genetics. *EMBO J* 20:4432–4442. <http://dx.doi.org/10.1093/emboj/20.16.4432>.
 57. Daniels RS, Downie JC, Hay AJ, Knossow M, Skehel JJ, Wang ML, Wiley DC. 1985. Fusion mutants of the influenza virus hemagglutinin glycoprotein. *Cell* 40:431–439. [http://dx.doi.org/10.1016/0092-8674\(85\)90157-6](http://dx.doi.org/10.1016/0092-8674(85)90157-6).
 58. Zaraket H, Bridges OA, Russell CJ. 2013. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus replication and pathogenesis in mice. *J Virol* 87:4826–4834. <http://dx.doi.org/10.1128/JVI.03110-12>.
 59. Ha Y, Stevens DJ, Skehel JJ, Wiley DC. 2002. H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. *EMBO J* 21:865–875. <http://dx.doi.org/10.1093/emboj/21.5.865>.
 60. Russell RJ, Gamblin SJ, Haire LF, Stevens DJ, Xiao B, Ha Y, Skehel JJ. 2004. H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology* 325:287–296. <http://dx.doi.org/10.1016/j.virol.2004.04.040>.
 61. Wiley DC, Skehel JJ. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem* 56:365–394. <http://dx.doi.org/10.1146/annurev.bi.56.070187.002053>.
 62. Skehel JJ, Wiley DC. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* 69:531–569. <http://dx.doi.org/10.1146/annurev.biochem.69.1.531>.
 63. Russell RJ, Kerry PS, Stevens DJ, Steinhauer DA, Martin SR, Gamblin SJ, Skehel JJ. 2008. Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion. *Proc Natl Acad Sci U S A* 105:17736–17741. <http://dx.doi.org/10.1073/pnas.0807142105>.
 64. Bodian DL, Yamasaki RB, Buswell RL, Stearns JF, White JM, Kuntz ID. 1993. Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. *Biochemistry* 32:2967–2978. <http://dx.doi.org/10.1021/bi00063a007>.
 65. Kumru OS, Joshi SB, Smith DE, Middaugh CR, Prusik T, Volkin DB. 2014. Vaccine instability in the cold chain: mechanisms, analysis and formulation strategies. *Biologicals* 42:237–259. <http://dx.doi.org/10.1016/j.biologicals.2014.05.007>.
 66. Wang W, Lu B, Zhou H, Suguitan AL, Jr, Cheng X, Subbarao K, Kemble G, Jin H. 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/

- 2004 vaccine virus in ferrets. *J Virol* 84:6570–6577. <http://dx.doi.org/10.1128/JVI.00221-10>.
67. Krenn BM, Egorov A, Romanovskaya-Romanko E, Wolschek M, Nakowitsch S, Ruthsatz T, Kiefmann B, Morokutti A, Humer J, Geiler J, Cinatl J, Michaelis M, Wressnigg N, Sturlan S, Ferko B, Batishchev OV, Indenbom AV, Zhu R, Kastner M, Hinterdorfer P, Kiselev O, Muster T, Romanova J. 2011. Single HA2 mutation increases the infectivity and immunogenicity of a live attenuated H5N1 intranasal influenza vaccine candidate lacking NS1. *PLoS One* 6:e18577. <http://dx.doi.org/10.1371/journal.pone.0018577>.
68. Cotter CR, Jin H, Chen Z. 2014. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. *PLoS Pathog* 10:e1003831. <http://dx.doi.org/10.1371/journal.ppat.1003831>.
69. Coenen F, Tolboom JT, Frijlink HW. 2006. Stability of influenza subunit vaccine. Does a couple of days outside the refrigerator matter? *Vaccine* 24:525–531. <http://dx.doi.org/10.1016/j.vaccine.2005.07.081>.
70. Hickey JM, Holtz KM, Manikwar P, Joshi SB, McPherson CE, Buckland B, Srivastava IK, Middaugh CR, Volkin DB. 2014. Mechanism of a decrease in potency for the recombinant influenza A virus hemagglutinin H3 antigen during storage. *J Pharm Sci* 103:821–827. <http://dx.doi.org/10.1002/jps.23848>.
71. Wang W, Suguitan AL, Jr, Zengel J, Chen Z, Jin H. 2012. Generation of recombinant pandemic H1N1 influenza virus with the HA cleavable by bromelain and identification of the residues influencing HA bromelain cleavage. *Vaccine* 30:872–878. <http://dx.doi.org/10.1016/j.vaccine.2011.11.101>.