Mutagenesis Studies of the H5 Influenza Hemagglutinin Stem Loop Region*

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Aleksandar Antanasijevic†, Arnab Basu§, Terry L. Bowlin§, Rama K. Mishra¶, Lijun Rong‖, and Michael Caffrey††

† From the ‡Department of Biochemistry and Molecular Genetics, University of Illinois, Chicago, Illinois 60607, §Microbiotix Inc., Worcester, Massachusetts 01605, the ¶Center for Molecular Innovation and Drug Discovery, Northwestern University, Evanston, Illinois 60208, and the ††Department of Microbiology and Immunology, University of Illinois, Chicago, Illinois 60612

Influenza outbreaks, particularly the pandemic 1918 H1 and avian H5 strains, are of high concern to public health. The hemagglutinin envelope protein of influenza plays a critical role in viral entry and thus is an attractive target for inhibition of virus entry. The highly conserved stem loop region of hemagglutinin has been shown to undergo critically important conformational changes during the entry process and, moreover, to be a site for inhibition of virus entry by antibodies, small proteins, and small drug-like molecules. In this work we probe the structure-function properties of the H5 hemagglutinin stem loop region by site-directed mutagenesis. We find that most mutations do not disrupt expression, proteolytic processing, incorporation into virus, or receptor binding; however, many of the mutations disrupt the entry process. We further assess the effects of mutations on inhibition of entry by a neutralizing monoclonal antibody (C179) and find examples of increased and decreased sensitivity to the antibody, consistent with the antibody binding site observed by x-ray crystallography. In addition, we tested the sensitivity of the mutants to MBX2329, a small molecule inhibitor of influenza entry. Interestingly, the mutants exhibit increased and decreased sensitivities to MBX2329, which gives further insight into the binding site of the compound on HA and potential mechanisms of escape. Finally, we have modeled the binding site of MBX2329 using molecular dynamics and find that the resulting structure is in good agreement with the mutagenesis results. Together these studies underscore the importance of the stem loop region to HA function and suggest potential sites for therapeutic intervention of influenza entry.

The membrane glycoprotein hemagglutinin (HA) plays a critical role in influenza infection (1). Antigenic properties are used to classify HA into 17 subtypes with H1, H5, and H7 being of special concern. For example, the 1918 pandemic H1N1 influenza outbreak caused more than 50 million deaths worldwide and seasonal influenza is responsible for greater than 250,000 deaths per year worldwide (2, 3). Moreover, the highly pathogenic avian influenza H5N1 and H7N9 have a >20% mortality rate and represent potential pandemic strains (4, 5). Current treatments for influenza include Tamiflu (oseltamivir) and Relenza (zanamivir), which target NA, and Symmetrel (amantadine) and Flumadine (rimantadine), which target the M2 channel (6). However, resistance is increasing in circulating influenza strains. For example, the 2008–2009 H1N1 strain of influenza exhibited ~100% resistance against Tamiflu (7). In summary, the consequences of influenza outbreaks and the observed resistance to current therapeutics strongly argue that new targets are urgently needed.

HA-mediated entry, as well as that of the analogous envelope proteins from Ebola, HIV, and SARS-CoV, occurs through binding to receptor and conformational changes that result in fusion of the viral and target cell membranes (8–10). HA are classified into 2 phylogenetic groups, based on sequence, structure, and immunogenicity, with H1 and H5 as examples of Group 1 HA and H3 and H7 as examples of Group 2 HA (11–13). HA is synthesized as a precursor and is subsequently cleaved to form a disulfide-linked complex consisting of HA1, the receptor binding subunit, and HA2, the subunit that mediates membrane fusion (1). In the first step of infection, HA in the neutral pH conformation binds to sialic acid receptors on the target cell surface. In the second step, the virus enters the endosome and the resulting low pH triggers a large conformational change in HA to expose a hydrophobic region, termed the fusion peptide. The newly exposed fusion peptide then inserts into the target membrane, thereby bringing the viral and target membranes in close contact to allow membrane fusion and entry of the virus into the cytoplasm (8). The critical role of HA in influenza entry renders it an attractive target for therapeutics designed to inhibit entry at either the binding or fusion steps (6). Interestingly, a number of fusion inhibitors have been shown to bind to the highly conserved stem loop region of HA, which is the region that undergoes a critical coil to helix tran-

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† To whom correspondence should be addressed: 900 S. Ashland, Chicago, IL 60607. Tel.: 312-996-4959; Fax: 312-413-0353; E-mail: caffrey@uic.edu.

‡ 100% resistance against Tamiflu (7).
Characterization of H5 Hemagglutinin Mutants

The hemagglutination assay was performed as previously described (21). Briefly, A549 lung cells, which were maintained in Dulbecco's medium with 10% FBS and 1% penicillin-streptomycin, were seeded to 2 × 10^4 cells/well of a 24-well cell culture plate in a volume of 0.5 ml. The following day, 500 μl of the virus stock was added to each of the wells of the A549 cells after removal of the medium. The plates were incubated at 37 °C in a CO_2 incubator. After ~6 h, the virosomes were aspirated and replaced with medium and the cells were allowed to rest for another 48 h. Luciferase activity was measured using the Luciferase Assay System from Promega and a Berthold FL12 lumimeter running Sirius software. In all cases, the viral entry levels fell within the linear range of detection (i.e. the values of the wild-type and mutants never exceeded 3 × 10^6 relative light units) (22). Entry levels were normalized to relative p24 levels, which were determined by ELISA as previously described (22). The inhibition of entry was performed by diluting stock solutions of C179 (1 mg/ml in 20 mM phosphate, 150 mM NaCl buffer, pH 7.4) or MBX2329 (20 mg in 1% DMSO) and, in the case of MBX2329, determining the IC_{50} from the equation: entry = entry_{max}/(1 + ([inhibitor]/IC_{50}) λ - n), where entry_{max} = entry in the absence of inhibitor and n = Hill coefficient.

Molecular Modeling—The apo crystal structure of the H5 (PDB code 2FK0) was considered for the modeling studies. The SITE-ID module of the Tripos molecular modeling package (23) was used to identify the potential small molecule ligand binding sites in the crystal structure. The FlexiDock docking package of Tripos was used to preposition the MBX2329 ligand in the possible ligand binding sites. FlexiDock works in torsional space, keeping the bond lengths and the angles constant while allowing the amino acids interacting with the ligand to be flexible during the docking process. The energetically most favorable position and the pose of MBX2329 obtained from FlexiDock was considered for further modeling studies. The molecular dynamics simulations (MDS) in the Optimized Potentials for Liquid Simulations (OPLS2005) force field was used to carry out the MDS of the H5 and MBX2329 complex structures. All MDS computations were carried out in MacroModel9.8 implemented in Schrodinger software suite (24) in one NPT ensemble (constant pressure and temperature). In the MacroModel dynamics panel, stochastic dynamics were chosen as it includes random forces that stimulate the buffering of a system by solvent molecules. To constrain the bond lengths to the original values, the “SHAKE” option was selected. The simulation of the complex was carried out at 300 K with a time step of 1.5 fs and equilibrium time of 1 ps. The MD simulation was run for 1, 5, and 10 ns recording the energies and the trajectories of the system. The plot of the potential energy versus the time at 10 ns revealed that the system had attained an equilibrium condition.

RESULTS

Mutant Sites—The stem loop is comprised of HA2 residues 40–110 (H3 numbering). In addition, we noted that residues 22–35 of HA1 and 17–25 of HA2 also make contacts in this

2 The abbreviation used is: MDS, molecular dynamics simulations.
region. Accordingly, we prepared 18 site-directed mutants at 14 different positions to probe the function of the stem loop region in H5 HA. The location and degree of conservation among diverse Groups 1 and 2 HA is shown in Fig. 1a. Positions Thr41, Gln42, Ile45, Asn53, and Leu99 of HA2 are highly conserved and designed to assay the importance of these residues to HA function. Positions Asp26, Ile28, and Met31 of HA1 and Thr49, Val52, Ser54, Ile55, Asp57, and Met102 of HA2 are less conserved and designed to assay differences between Group 1 (i.e. H1 and H5) and Group 2 (i.e. H3 and H7) HA. The location of the mutated side chains with respect to the HA structure is shown in Fig. 1b with HA1 colored blue and HA2 colored red. For clarity, only one monomer of the symmetric trimer is shown. Note that the residues range from being highly solvent exposed to being relatively buried and that the mutated residues are involved in a variety of intra and intermolecular contacts, which will be discussed below.

**Mutant Expression, Processing, Incorporation into Virions, and Receptor Binding**—We first assessed the mutational effects on protein expression, processing (i.e. cleavage of HA0 to HA1 and HA2), and incorporation into virions using a Western blot analysis of wild-type and mutant virions. The results of this analysis is shown in Fig. 2a using a monoclonal antibody to HA1, which will detect processed HA1 and unprocessed HA0, and summarized in Table 1. In the case of the wild-type, both HA0 and HA1 are observed with the majority of the HA found in virions observed to be processed (∼85%). In the case of the mutants all mutants exhibited >50% HA processing. On the other hand, mutants HA1-D26K, HA1-M102L, HA2-V52A, and HA2-I55A exhibit significantly reduced levels of total HA (<30% of the wild-type HA levels), suggesting that they have significantly reduced the expression and/or incorporation of HA into virions. In contrast, all other mutants exhibit HA levels that are similar to the wild-type, suggesting that the mutation has not significantly affected HA expression, processing, and incorporation into virions. We next used the hemagglutination assay to assess mutational effects on the HA1 conformation and the ability to bind receptor. In this assay, virion preparations are added to red blood cells (RBC) placed at the bottom of the well and binding of virions to RBC is then visualized as the agglutination of the RBC. As shown in Fig. 2b (and summarized in Table 1) all virion preparations cause agglutination of the RBC with mutants HA1-I28A, HA1-I28V, HA1-M31A, HA2-T41A, HA2-Q42A, HA2-I45A, HA2-V52A, HA2-N53A, HA2-S54R, HA2-I55A, HA2-I55V, HA2-D57E, HA2-L99A, and HA2-M102A exhibiting wild-type titers (agglutination observed up to ∼1:16 dilution) and HA1-D26K, HA2-T49A, and HA2-M102L exhibiting lower titers (agglutination observed up to...
Thus, with the possible exceptions of mutants HA1-D26K, HA2-T49A, and HA2-M102L, the mutant HA are competent to bind to their receptor and have presumably not disrupted the HA1 structure.

Mutant Effects on HA-mediated Entry—Previously, we and others have shown that virus-like particles containing envelope protein on an HIV background vector are useful surrogates to characterize the functional properties of envelope proteins from diverse viruses including influenza, HIV, Ebola, and SARS-CoV (20, 25–27). In Fig. 3 (and summarized in Table 1) we show the relative entry of virions containing wild-type or single site mutations into A549 lung cells. First note that all single-site mutations exhibit decreased entry with respect to the wild-type, even those with relatively conservative substitutions (e.g., HA2-T49A or HA2-D57E). For ease of discussion we have divided the effects into 3 classes: non-functional (< 1% entry), impaired (1–25%), and intermediate (> 25%). Interestingly, there are only 2 mutants that fall into the non-functional category: HA1-D26K and HA2-I55A. Presumably, the very low entry levels of these mutants is due in part to their significantly low levels of expression and/or incorporation into virions. Mutants that exhibit impaired (but measurable) entry include: HA1-I28A, HA1-I28V, HA1-M31A, HA2-T41A, HA2-Q42A, HA2-I45A, HA2-T49A, HA2-N53A, HA2-L99A, HA2-M102A, and HA2-M102L. Finally, mutants that exhibit the least effects on entry include: HA1-M31L, HA2-V52A, HA2-S54R, HA2-I55V, and HA2-D57E. Taken together, the mutational effects on entry underscores the importance of this region of the stem loop region to HA-mediated entry.

Mutant Effects on Entry Inhibition by Monoclonal Antibody C179—Previously, the monoclonal antibody C179 has been shown to neutralize the entry of influenza virus (14) by binding to the HA stem loop region and stabilizing the neutral pH conformation (15). Accordingly, we were interested in testing whether our stem loop region mutants would affect the binding and entry inhibition of C179. In this case we used the viral entry

~1/4 dilution). Thus, with the possible exceptions of mutants HA1-D26K, HA2-T49A, and HA2-M102L, the mutant HA are competent to bind to their receptor and have presumably not disrupted the HA1 structure.

Mutant Effects on HA-mediated Entry—Previously, we and others have shown that virus-like particles containing envelope protein on an HIV background vector are useful surrogates to

Characterization of H5 Hemagglutinin Mutants

![Western blot and hemagglutination assay of virus-like particles of H5 HA wild-type and mutants.](image-url)

(a) Western blot analysis of HA in virus-like particles with no env corresponding to virions without HA and NA. Note that the primary antibody used is against HA1 and thus detects both HA0 and HA1. b, hemagglutination assay of virus-like particles with “NA” and “no HA/NA” samples corresponding to virions prepared with only NA and without NA or HA, respectively. Each assay was performed in triplicate.

| Mutant† | WT entry | HA expression | HA processing | Hemagglutination |
|---------|----------|---------------|---------------|-----------------|
| WT | 100 ± 1 | 100 | 85 | 1:16 |
| HA1-D26K | 0.10 ± 0.07 | 28 | 50 | 1:4 |
| HA1-I28A | 4.4 ± 0.8 | 100 | 73 | 1:32 |
| HA1-I28V | 23 ± 3 | 83 | 79 | 1:16 |
| HA1-M31A | 2.8 ± 1.3 | 56 | 74 | 1:16 |
| HA1-M31L | 51 ± 13 | 27 | 96 | 1:16 |
| HA2-T41A | 17 ± 9 | 150 | 69 | 1:32 |
| HA2-Q42A | 14 ± 2 | 62 | 77 | 1:8 |
| HA2-I45A | 7.4 ± 1.1 | 39 | 84 | 1:32 |
| HA2-T49A | 19 ± 5 | 71 | 64 | 1:4 |
| HA2-V52A | 51 ± 12 | 20 | 100 | 1:8 |
| HA2-N53A | 2.1 ± 1.5 | 130 | 75 | 1:8 |
| HA2-S54R | 47 ± 5 | 82 | 79 | 1:8 |
| HA2-I55A | 0.7 ± 0.9 | 27 | 56 | 1:8 |
| HA2-I55V | 67 ± 5 | 120 | 74 | 1:16 |
| HA2-D57E | 33 ± 7 | 100 | 75 | 1:32 |
| HA2-L99A | 5.8 ± 1.2 | 97 | 73 | 1:16 |
| HA2-M102A | 11 ± 3 | 37 | 75 | 1:4 |
| HA2-M102L | 51 ± 12 | 20 | 100 | 1:8 |
| No env | 0.90 ± 0.30 | | | |

† Numbering according to H3 HA.
‡ % HA expression with respect to the wild-type.
§ % Processing with respect to total HA.
∥ Largest dilution at which agglutination was observed.
assay under conditions where wild-type HA is only partially inhibited (Fig. 4 and summarized in Table 2). For this analysis mutants HA1-D26K and HA2-I55A were excluded due to their very low entry levels in the absence of entry inhibitor (Fig. 3 and Table 1). As shown in Fig. 4, under these conditions C179 reduced wild-type entry to 29 ± 3%. Mutants HA2-T41A, HA2-Q42A, HA2-T49A, HA2-V52A, HA2-N53A, HA2-S54R, HA2-D57E, HA2-M102A, and HA2-M102L exhibit entry inhibition that is similar to the wild-type (<20% different, Table 2), suggesting that their stem loop structures are competent to bind the antibody. In contrast, HA2-L99A is more sensitive to entry inhibition by C179 (300% more sensitive, Table 2), suggesting that the mutation enhances antibody binding and/or mode of action. Interestingly, mutants HA1-I28V, HA1-M31A, HA1-M31L, HA2-I45A, and HA2-I55V render HA less sensitive to entry inhibition by C179 (>200% less sensitive, Table 2) suggesting that the mutation decreases antibody binding and/or mode of action.

Mutant Effects on Entry Inhibition by MBX2329—Recently, our groups have described the inhibitory properties of MBX2329, a small drug-like molecule, to diverse Group 1 strains of HA (20). Based on competition assays with antibody C179, MBX2329 binds to the stem loop region of HA (20) and thus we were interested in testing whether our stem loop region mutations had an effect on the binding and inhibition properties of the compound. Accordingly, we determined the IC50 of wild-type and mutants for MBX2329 with the goal of gaining insight into the binding site of the compound. As shown in Fig. 5 and summarized in Table 2, the mutants exhibit diverse effects. Mutants HA1-I28V, HA2-T41A, HA2-Q42A, HA2-M265V, and HA2-L99A render HA less sensitive to MBX2329 inhibition, while mutants HA1-D26K, HA1-M31A, and HA2-M102A render HA more sensitive to MBX2329 inhibition. These results suggest that the stem loop region mutations affect the interaction between MBX2329 and HA, potentially altering the binding site or mode of action of the compound.
Characterization of H5 Hemagglutinin Mutants

T49A, HA2-I55V, HA2-D57E, and HA2-M102L have little effect on the IC$_{50}$ of the compound. Interestingly, mutants HA1-128A, HA1-M31L, and HA2-M102A have enhanced the inhibitory properties of MBX2329 and thus the mutations appear to improve the binding and/or mode of action of the compound. On the other hand, mutants HA2-I45A, HA2-V52A, HA2-N53A, HA2-S54R, and HA2-L99A become insensitive to MBX2329 and thus the mutation appears to have disrupted the binding and/or mode of action of the compound. Note that this latter set of mutations may give insights into potential escape mechanisms for this compound.

Molecular Modeling of MBX2329 to the H5 HA Stem Loop Region—In a next step, we analyzed the binding pose of MBX2329 with the HA stem loop region obtained from the 10-ns MDS. Importantly, the docking followed by the MDS was performed without any input from the mutagenesis data. The MBX2329 ligand binding site is found to be in the similar location to that of the TBHQ site within the stem loop (18). The binding pose of compound and its interaction with the different residues of HA stem is shown in Fig. 6. Notably, MBX2329 shows interactions with the side chains of HA1-Gln$^{40}$, HA1-Thr$^{31}$, HA2-Ile$^{45}$, HA2-Val$^{48}$, HA2-Thr$^{49}$, HA2-Val$^{52}$, and HA2-Asn$^{53}$.

DISCUSSION

In this work we generated 18 site-directed mutations to 14 conserved sites in the H5 HA stem loop region. In the case of 13 mutations, they had very little effect on HA expression, processing, incorporation into virions, and binding to receptor. On

| Mutant   | C179 Inhibition | C179 Potency$^a$ | MBX2329 IC$_{50}$ | MBX2329 potency |
|----------|-----------------|-----------------|------------------|-----------------|
| WT       | 29 ± 3          | 1.0             | 4.4 ± 0.6        | 1.0             |
| HA1-D26K | ND$^b$          |                 |                  |                 |
| HA1-I28A | 49 ± 5          | 0.6             | 0.29 ± 0.09      | 15              |
| HA1-I28V | 26 ± 3          | 1.1             | 0.6 ± 1.0        | 0.7             |
| HA1-M31A | 66 ± 16         | 0.4             |                  |                 |
| HA1-M31L | 46 ± 8          | 0.6             | 0.23 ± 0.06      | 19              |
| HA2-T41A | 21 ± 4          | 1.4             | 3.5 ± 0.9        | 1.3             |
| HA2-Q92A | 35 ± 6          | 0.8             | 4.0 ± 0.7        | 1.1             |
| HA2-H5A  | 88 ± 15         | 0.3             | 50 ± 6           | 0.1             |
| HA2-T49A | 31 ± 1          | 0.9             | 5.1 ± 0.9        | 0.9             |
| HA2-V52A | 38 ± 3          | 0.8             | >50              | <0.1            |
| HA2-N53A | 37 ± 10         | 0.8             | >50              | <0.1            |
| HA2-S54R | 32 ± 3          | 0.9             | >50              | <0.1            |
| HA2-I55A | ND$^b$          |                 |                  |                 |
| HA2-I55V | 59 ± 4          | 0.5             | 6.8 ± 0.9        | 0.6             |
| HA2-D57E | 27 ± 3          | 1.1             | 6.0 ± 1.0        | 0.7             |
| HA2-L99A | 9.3 ± 1.1       | 3.1             | >50              | <0.1            |
| HA2-M102A| 37 ± 3          | 0.8             | 1.2 ± 0.3        | 3.7             |
| HA2-M102L| 37 ± 9          | 0.8             | 2.4 ± 0.3        | 1.8             |

$^a$ Potency is defined as WT inhibition/mutant inhibition.

$^b$ ND, not determined.

FIGURE 5. Mutant effects on entry inhibition of H5 HA by the small molecule MBX2329. The relative entry levels are based of the entry of pseudovirus entry into A549 lung cells with a luciferase-based assay in the presence of varying amounts of MBX2329 and the IC$_{50}$ and error values were determined as described under “Experimental Procedures.”

FIGURE 6. Molecular dynamics model of the MBX2329 interaction with H5 HA. MBX2329 is shown in the space-filling representation and the H5 HA side chains in closest proximity to the compound are highlighted in yellow.
A number of mutations have previously been characterized for HA at the identical sites. For example, Reed et al. (29) have characterized the equivalent HA2-S54R mutant in H5 HA and found that the mutation significantly disrupted entry and fusion, an observation that is in agreement with the results presented in this work (cf. Table 1). On the other hand, Qiao et al. (30) found that HA2-V55A was competent for fusion, which is somewhat different from our result (cf. Table 1) and most probably due to subtle differences in the sequence and structure of H3, a Group 2 HA, and H5, a Group 1 HA. In another study, Casali et al. (31) characterized synthetic peptides corresponding to part of the region under study in this work and found that the HA2-E57Q substitution significantly decreased the native helical content at both neutral and acidic pH underscoring the importance of this residue to HA structure and function, an observation in agreement with the decreased entry of our HA2-D57E mutant (cf. Table 1).

Finally it is next of interest to consider the mutational effects with respect to the H5 HA structure. In Fig. 7 we show space filling diagrams of the effects on entry, C179 entry inhibition and MBX2329 entry inhibition. In this representation, we have
Characterization of H5 Hemagglutinin Mutants

considered the outer face (i.e. solvent exposed) and inner face (i.e. contact region with other subunits) of a single HA monomer. The red, blue, and green coloring scheme corresponds to negative, minimal, and enhanced effects on HA-mediated entry, respectively. In the case of the mutational effects on virus entry, substitutions to HA2-Ile45 and HA2-Asn53 of the HA outer face and HA1-Ile28, HA1-Met31, and HA2-Leu99 of the HA inner face had the largest effects (red color, entry <10% of the wild-type). Note that during entry, the depicted inner residues are not in a region that undergoes a large conformational change (8, 9) and thus the mutational effects most likely arise from perturbation of the beginning neutral pH structure. In contrast, the depicted outer residues are in a region that undergoes a large conformational change (8, 9) and thus mutational effects could arise from perturbation of the beginning structure (i.e. the neutral pH conformation) and/or later structures (i.e. the low pH conformations).

In the case of the mutational effects on entry inhibition by the monoclonal antibody C179, substitutions to HA2-Ile45 of the outer face and HA1-Met31 and HA2-Asn53 of the inner face decreased potency to ≤ 0.5 with respect to the wild-type (Fig. 7, red color). In contrast, substitution of HA2-Leu99 enhanced potency of the antibody to > 3 with respect to the wild-type (green color). With respect to the neutral and low pH conformations of HA, C179 binds to the neutral pH conformation shown in Fig. 7 (15). Consequently, the mutational effects of the residues depicted on the outer face are presumably due to direct interactions with the antibody and the mutational effects of the residues depicted on the inner face are presumably indirect and propagated effects.

In the case of the mutational effects on entry inhibition by the small molecule MBX2329, substitutions to HA2-Ile45, HA2-Val52, HA2-Asn53, and HA2-Ser54 of the outer face and HA2-Leu99 of the inner face had the largest effects (Fig. 7, red color, potency ≤ 0.1 with respect to the wild-type). Importantly, these residues are highly conserved in the Group 1 HA (e.g. H1 and H5, Fig. 1a), which is the group that MBX2329 inhibits. On the other hand, substitutions to HA1-Ile28, HA1-Met31, and HA2-Met109 of the inner face significantly enhanced entry inhibition by MBX2329 (green color, potency = > 3). With respect to the neutral and low pH conformations of HA, we suggest that the mutational effects depicted for the outer face residues directly affect the binding of MBX2329 to the neutral pH conformation, which is in agreement with the molecular dynamics model shown in Fig. 6, and the mutational effects depicted for the inner face residues to be indirectly affecting the binding of the compound to the neutral pH conformation. Nonetheless, more subtle mutational effects to the low pH conformations are possible. Finally, residues HA1-Met31, HA2-Ile45, and HA2-Leu99 exhibited large effects in all three assays, suggesting that they may represent especially sensitive regions within the HA stem loop region. Notably, HA2-Ile45 is highly conserved among all HA subtypes (Fig. 1) and is located on the outer solvent-exposed face of HA (Fig. 7).

In summary, the mutations presented herein underscore the importance of the stem loop region to HA function. Importantly, many of the sensitive sites of the HA stem loop region are highly conserved and solvent exposed and, moreover, they may represent novel target sites for therapeutic intervention of influenza entry. Such sites may be of particular importance in targeting new pandemic outbreaks of influenza including those of avian H5 and H7 HA.

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