Analysis of residues near the fusion peptide in the influenza hemagglutinin structure for roles in triggering membrane fusion

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Abstract

Influenza virus entry occurs in endosomes, where acidification triggers irreversible conformational changes of the hemagglutinin glycoprotein (HA) that are required for membrane fusion. The acid-induced HA structural rearrangements have been well documented, and several models have been proposed to relate these to the process of membrane fusion. However, details regarding the role of specific residues in the initiation of structural rearrangements and membrane fusion are lacking. Here we report the results of studies on the HA of A/Aichi/2/68 virus (H3 subtype), in which mutants with changes at several ionizable residues in the vicinity of the “fusion peptide” were analyzed for their effects on the pH at which conformational changes and membrane fusion occur. A variety of phenotypes was obtained, including examples of substitutions that lead to an increase in HA stability at reduced pH. Of particular note was the observation that a histidine to tyrosine substitution at HA1 position 17 resulted in a decrease in pH at which HA structural changes and membrane fusion take place by 0.3 relative to WT. The results are discussed in relation to possible mechanisms by which HA structural rearrangements are initiated at low pH and clade-specific differences near the fusion peptide.

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Keywords: Influenza; Hemagglutinin; Membrane fusion; Fusion peptide; HA structure; Conformational change

Introduction

Membrane fusion is a biological process required for a variety of fundamental viral and cellular functions. Developments in recent years have significantly expanded the understanding of the fusion mechanisms for both class I and class II viral fusion glycoproteins (Barnard et al., 2006; Colman and Lawrence, 2003; Doms, 2004; Dutch et al., 2000; Earp et al., 2005; Eckert and Kim, 2001; Harrison, 2005; Huang et al., 2003; Kielian, 2006; Lamb et al., 2006; Sieczkarski and Whittaker, 2005; Skehel and Wiley, 2000; Smith and Helenius, 2004). Among the class I viral fusion proteins, related mechanisms for fusion have evolved in which a metastable form of the molecule is converted into a highly thermostable conformation during the fusion process. These thermostable structures are rod-like in appearance and all contain a central trimeric α-helical coiled coil and antiparallel polypeptide chains that pack against it. As a consequence of the refolding events, the hydrophobic transmembrane and fusion peptide domains of the glycoproteins, which are postulated to associate with the viral and cellular membranes, respectively, as part of the fusion process, are brought into close proximity with one another. For the class I viral fusion proteins, there are a number of mechanisms by which such conformational changes can be triggered to initiate the fusion process. These include receptor binding with the involvement of coreceptors, receptor binding and interaction with separate viral fusion proteins, and activation of a viral fusion protein by acidification following endocytosis.

Influenza A is a well-characterized example of a virus that enters the host cell via the endocytic pathway, and structural studies spanning the past three decades have made the HA
glycoprotein a valuable paradigm for studies on viral membrane
fusion in general (Bullough et al., 1994; Chen et al., 1998; Wilson et al., 1981). Similar to other class I viral fusion pro-
teins, the polypeptide chains of the HA precursor (HA0) 
associate as homotrimers in the endoplasmic reticulum during
biosynthesis. Each monomer of HA0 is subsequently cleaved at
a surface loop into the disulfide-linked polypeptides HA1 and
HA2. This generates hydrophobic HA2 N-terminal fusion
peptide domains and transforms the molecule into its metastable
conformation to activate the membrane fusion potential of
HA. As a consequence, HA0 cleavage is required for virus
infectivity (Appleyard and Maber, 1974; Klenk et al., 1975;
Lazarowitz and Choppin, 1975). After attachment to host cells
and internalization, HA undergoes irreversible conformational
changes due to the acidification of the endosomal environment,
and membrane fusion is induced.

The current study focuses on the analysis of residues near the
fusion peptide of cleaved HA that may be involved in the
initiation of the acid-induced HA conformational changes re-
quired for fusion. While it is known that amino acid substi-
tutions at various locations in the HA trimer are capable of
destabilizing the native structure leading to an increase in the
pH of fusion, the possibility that protonation of specific resi-
dues provide the initial trigger for conformational changes
remains unresolved. The region surrounding the fusion peptide
in the native HA structure is of interest regarding potential
triggers for fusion for several reasons. Among the many
mutants with elevated fusion pH identified in studies based on
amanadine resistance, site-directed mutagenesis, and reverse
genetics, those involving amino acid substitutions in and
around the fusion peptide are particularly well represented
(Cross et al., 2001; Daniels et al., 1985; Lin et al., 1997; Stein-
hauer et al., 1993). In fact, nearly all amino acid substitutions in
the N-terminal region of the fusion peptide that have been
analyzed to date lead to increased fusion pH, regardless of the
HA position or the amino acid introduced (Cross et al., 2001;
Gething et al., 1986; Steinhauser et al., 1995). Studies on double
mutant HAs also indicate that amino acid substitutions in and
around the fusion peptide are dominant in dictating the pH of
fusion when expressed in combination with substitutions
elsewhere in the molecule (Steinhauser et al., 1996). Other
studies using anti-peptide antibodies to detect changes in HA
structure also suggest that conformational changes involving
the fusion peptide and proximal residues precede the decay-
imerization of the HA1 head domains (White and Wilson,
1987).

When HA0 is cleaved into HA1 and HA2 to prime membrane
fusion potential, only six residues at the C-terminus of HA1
and 12 residues at the N-terminus of HA2 are relocated (Chen et al.,
1998) (Fig. 1). As the conformational changes that accompany
cleavage are restricted to this region, the accessibility to solvent
is altered for only a selected number of ionizable residues in the
trimer. A comparison of the structures of different subtype HAs
shows that some of the ionizable residues that are buried by the
fusion peptide after cleavage are completely conserved, while
others vary strictly along clade-specific lines (Gamblin et al.,
2004; Ha et al., 2002; Russell et al., 2004; Wilson et al., 1981).

The recently determined H13 subtype HA structure (Russell et
al., unpublished) now divides the HAs into five clades, and when
structural characteristics of the fusion peptide region are com-
pared the five clades can be separated into two groups as depicted in
Fig. 2. The H1 group includes H1-, H9-, and H13-
like viruses, and the H3 group includes the H3 and H7 clades. In
all HAs HA2 residues K51, D109, and D112 are completely
conserved, whereas the residues at positions HA1 17, and HA2
106, and 111 are group specific. Among H3 group HAs, such as
the Aichi HA analyzed in this study, HA1 17 and HA2 106 are
nearly always histidine, and HA2 111 is threonine. In the H1
group HAs, HA1 17 is tyrosine, HA2 111 is histidine, and HA2
106 is either a basic lysine or arginine residue (Fouchier et al.,
2005; Kawaoka et al., 1990; Nobusawa et al., 1991; Rohm et al.,
1996).

We postulate that these residues are involved in modulating the
stability of cleaved HA, and that the introduction of substitu-
tion mutations at these positions will have a tendency to
alter the pH at which conformational changes are induced.
Changes to positions that result in increased stability (reduced
pH of fusion) could possibly help identify residues that are
involved in the triggering of conformational changes when
protonated. To examine the possible role of specific residues in
the initiation of fusion upon acidification, we generated a
series of single, double, and triple amino acid substitution
mutants in an H3 subtype HA. The mutant HAs were analyzed
for folding, cell-surface transport, acid-induced structural
changes, the pH at which they take place, and membrane
fusion properties. A variety of phenotypes were detected, in-
cluding examples that undergo conformational changes and
mediate membrane fusion at reduced pH. A possible role for
particular ionizable residues in the initiation of the fusion
process is discussed. Furthermore, we compare the structures
of this region among HA subtypes and discuss clade-specific
differences with regard to acid-induced activation of mem-
bane fusion.
Results

A series of single, double, and triple amino acid substitution mutants were constructed in the HA of A/Aichi/2/68 virus at position 17 of the HA1 subunit and at positions 51, 105, 106, 109, 111, and 112 of the HA2 subunit, and recombinant vaccinia viruses were generated for their expression. The structural locations of these positions are indicated in Fig. 2. These include residues that become occluded from access to bulk solvent following cleavage of HA0 and transition to the metastable neutral pH conformation. For these, the chemical environment and potential for protonation may change following cleavage activation of HA. The mutants that were analyzed involved alanine substitutions, and changes in which the ionization properties of side chains were altered using amino acids of similar dimension. In addition, we made mutants in which the residues of the H3 HA at positions 17 of HA1 and positions 105, 106, and 111 of HA2 were exchanged for residues at the equivalent structural locations in the H1 group HAs.

Cell surface expression by ELISA

The mutants that were generated and analyzed in this study are listed in Table 1, along with ELISA data for antibody
reactivity to cell-surface expressed HAs. A panel of six monoclonal antibodies and a rabbit polyclonal antiserum were used to assess HA expression and protein folding following infection of HeLa cell monolayers with recombinant vaccinia viruses. The monoclonal antibodies utilized are known to bind to a range of sites on the HA structure based on the locations of changes mapped in neutralization-resistant virus mutants (Daniels et al., 1983) and electron microscopy and X-ray crystallography studies of HA-antibody complexes (Bizebard et al., 2001; Wrigley et al., 1983). The monoclonal antibodies HC3 and HC100 recognize the neutral as well as the low pH conformation of HA. The other monoclonal antibodies are specific for the neutral pH structure of HA but lose reactivity as a result of acid-induced HA conformational changes. The \( \alpha \)-X31 antibody is a rabbit polyclonal serum raised against the bromelain-solubilized ectodomain (BHA) of the WT HA of A/Aichi/2/68 virus.

Table 1

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<th></th>
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<th>HC68</th>
<th>HC100</th>
<th>HC263</th>
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<td>90</td>
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<td>96</td>
<td>106</td>
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<td>H17/A, H1062/A, T1112/A</td>
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<tr>
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<td>31</td>
<td>12</td>
<td>27</td>
<td>91</td>
<td>23</td>
<td>96</td>
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</tbody>
</table>

Values represent averages derived from a minimum of three separate experiments.

Influenza HA is expressed at the surface of recombinant vaccinia virus-infected cells in its uncleaved precursor form. Therefore, cleavage of HA0 into the HA1 and HA2 polypeptides by trypsin treatment of HA-expressing cell monolayers can be

Table 2

<table>
<thead>
<tr>
<th>HA Expression characteristics</th>
<th>HA</th>
<th>Trypsin cleavage</th>
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<tbody>
<tr>
<td>WT, all single mutants, (H1062/A, T1112/A), (H1062/F, T1112/V), and (H17/Y, H1062/A, T1112/A)</td>
<td>ELISA—react with non-specific antibodies, react with conformation-specific antibodies</td>
<td>Trypsin cleavage—HA0 cleaved, HA1 generated, HA2 generated</td>
</tr>
<tr>
<td>Interpretation—HA expressed on cell surface in native neutral pH conformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H1062/R, T1112/H), (H17/A, H1062/A, T1112/A), and (H17/Y, H1062/R, T1112/H)</td>
<td>ELISA—react with non-specific antibodies, reduced reactivity with conformation-specific antibodies</td>
<td>Trypsin cleavage—HA0 doublet (CHO?) cleaved, HA1 degraded, HA2 generated, also present in small quantities</td>
</tr>
<tr>
<td>Interpretation—HA expressed on cell surface in non-native conformation, possibly resembling the low pH structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(D1092/A, D1122/A)</td>
<td>ELISA—poor reactivity to all antibodies</td>
<td>Trypsin cleavage—HA0 bands only, with or without trypsin</td>
</tr>
<tr>
<td>Interpretation—HA does not fold correctly or is very unstable, degraded in the ER or during transport to the cell surface</td>
<td></td>
<td></td>
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</tbody>
</table>

* Comments and interpretations based on Table 1 and Fig. 3.
Fig. 4. Graphs of ELISA data showing the pH of conformational change for various mutant and WT HAs. Graphs plot the ratios of HC67/HC3 reactivity as a function of pH. HC67 binds well with neutral pH HA, but poorly to the low pH structure. HC3 binds equally well with both HA conformations.
used as an assay for HA transport to the cell surface. In addition, the characteristic migration patterns of the trypsin cleavage products following polyacrylamide gel electrophoresis under reducing conditions can provide an indicator that the HA is folded properly in the native conformation. Fig. 3 shows a western blot analysis of WT and mutant HA-expressing cell lysates processed following the incubation of monolayers with or without trypsin. The results reveal that WT HA and all single residue substitution mutants can be cleaved into HA1 and HA2 polypeptides of characteristic size, confirming that they are expressed at the cell surface. Digestion of the double mutants (H1062R, T1112H) and (D1092A, T1112V) and the triple mutant (H171Y, H1062A, T1112A) also yielded HA1 and HA2 polypeptides that resembled in size those of WT HA, although the (H1062F, T1112V) HA2 band migrated as a doublet containing an additional product of lower apparent molecular weight. On the other hand, the results obtained following trypsin treatment of the double mutants (H1062R, T1112H) and (D1092A, D1122A), and the (H171Y, H1062A, T1112A) and (H171Y, H1062R, T1112H) triple mutants differed significantly from those observed with WT HA. The (D1092A, D1122A) HA blot revealed only low intensity bands corresponding to HA0, with or without added trypsin. Presumably, this HA does not fold correctly or express on the cell surface, as suggested by the lack of reactivity to any sera by ELISA in experiments described above. The other three mutant HAs, (H1062R, T1112H), (H171Y, H1062A, T1112A), and (H171Y, H1062R, T1112H), produce bands corresponding to HA2 in molecular weight following trypsin digestion, but no HA1 bands can be detected. For these mutants, a slower migrating HA0 component is observed in the absence of added protease, suggesting aberrant glycosylation properties. The ELISA results described above demonstrate that polyclonal sera and antibodies that recognize both neutral and low pH HA can effectively bind these three mutant HAs, but they are recognized poorly with the neutral pH-specific antibodies. Taken together, the results of the two assays suggest that these mutants are likely to be transported to the cell surface in an altered conformation, perhaps resembling the low pH structure. The results and our interpretations of the ELISA and cell surface trypsin cleavage experiments are summarized in Table 2.

Analysis of the pH of conformational change by ELISA

Antibody HC3 recognizes residues located in a surface loop that does not change structure when HA is acidified (Bizebard et al., 1995; Wiley and Skehel, 1987) and therefore reacts well with both the neutral and low pH structures of HA. On the other hand, HC67 recognizes an antigenic site at a membrane distal trimeric interface that is lost when the HA head regions de-trimerize at low pH. Consequently, pH adjustment of HA-expressing cells followed by ELISA to compare the reactivity of these two antibodies can be used to estimate the pH at which conformational changes take place. Fig. 4 shows the ELISA results for the single amino acid HA substitution mutants presented in graph form plotting the ratio of HC67 to HC3 reactivity as a function of pH. The midpoint of the curves shown in Fig. 4 is designated as the pH of conformational change and the differences between these determinations for mutant HAs compared to WT (the ΔpH of conformational change) are shown as a component of Table 3. Several of the mutant HAs demonstrated a significantly elevated pH of conformational change, while others appeared more acid-stable. Most notable amongst the latter were the HA1 H17Y and the HA2 K51A mutants, which were observed to undergo conformational changes at a pH 0.3 lower than WT (pH 4.9 vs. pH 5.2).

Analysis of the pH of conformational change by trypsin susceptibility

Once HA0 has been cleaved into HA1 and HA2, the neutral pH structure is highly resistant to further proteolytic digestion by trypsin and several other proteases. However, the molecular rearrangements induced by acidification render the HA1 subunit susceptible to digestion by trypsin (Skehel et al., 1982). Therefore, we used trypsin susceptibility as an alternative assay to determine the pH at which the structural changes take place for mutant HAs. CV1 cells were infected with recombinant vaccinia viruses and the HA-expressing cell monolayers were trypsin treated to cleave HA0, the pH was adjusted in decreasing increments of 0.2, and HA digestion was analyzed following a second trypsin digestion. Cell lysates were then processed using reducing conditions and analyzed by western blot. The digestion profiles for a selection of the HAs as a function of pH are shown in Fig. 5. Band intensities were quantified to estimate the pH of HA1 digestion as an indicator of the pH of conformational change, and the ΔpH values relative to WT are shown in Table 3. Overall, the results are in general agreement with those obtained by ELISA, with only a few examples of minor variation in the values obtained for ΔpH of conformational change.

Table 3

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ΔpH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ELISA</th>
<th>Trypsin</th>
<th>Polykaryon</th>
<th>Average</th>
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</tbody>
</table>

<sup>a</sup> The values given for each assay represent the average from a minimum of three experiments. In all cases, the range among replicates was less than 0.1 pH units.

<sup>b</sup> No polykaryon formation detected at any pH.

<sup>c</sup> Only low levels of fusion detected, and only at pH 0.1 or more lower than WT.
Fig. 5. Western blot analysis for the determination of the pH of conformational change by trypsin susceptibility. HA expressing cells were treated with trypsin to cleave HA0, washed, pH was adjusted as indicated, and monolayers were again treated with trypsin. Lysates were then analyzed by western blot following SDS–PAGE under reducing conditions. The digestion of HA1 bands is indicative of the pH of conformational change.

Fig. 6. Polykaryon formation by HA-expressing BHK cells following incubation at the indicated pH.
Analysis of the pH of membrane fusion by polykaryon formation

The fusion properties of recombinant vaccinia virus-infected cells expressing WT or mutant HAs were analyzed using an assay for polykaryon formation. BHK-21 cells were infected with recombinant vaccinia viruses, trypsin treated, the pH was reduced incrementally from one well to the next, and the pH of monolayers was neutralized. Cells were then incubated in complete medium and monitored for evidence of fusion by light microscopy. The results of polykaryon formation as a function of pH are shown for selected mutants in Fig. 6, and the ΔpH results for all mutants are summarized in Table 3. The results for pH of polykaryon formation are consistent with those obtained by ELISA and trypsin susceptibility for pH of conformational change. The only clear discrepancy involved the double mutants at HA2 positions 106 and 111. The (H1062A, T1112A) and (H1062F, T1112V) mutants were both observed to undergo structural changes at pH 0.4 above WT, but in both cases the capacity to mediate polykaryon formation was found to be inhibited. No fusion activity was observed with (H1062A, T1112A) HA-expressing cells, and with cells expressing the (H1062F, T1112V) mutant, low levels of polykaryon formation were detected only at pH 0.1 or more below WT HA. HA2 residues 106 and 111 are at the ends of the peptide segment of the neutral pH coiled coil that undergoes a helix-to-loop transition upon acidification. Therefore, the changes at these positions may affect this particular molecular rearrangement or the formation of a low pH structure that is optimal for fusion in addition to causing decreased stability of HA and increased pH of conformational change as detected by ELISA and trypsin assays. No fusion activity was detected for trypsin-treated non-recombinant vaccinia viruses, or for non-trypsin-treated HA-expressing cells (data not shown). The results demonstrate that relative to WT HA, the pH of fusion as measured by polykaryon formation is consistent with the ΔpH data obtained using the conformational change assays (Table 3).

Discussion

A number of HA mutants that undergo acid-induced conformational changes and mediate membrane fusion at elevated pH have been reported (Cross et al., 2001; Daniels et al., 1985; Doms et al., 1986; Gething et al., 1986; Lin et al., 1997; Steinhauer et al., 1993, 1995). The mutations responsible for this phenotype have been mapped to various locations throughout the length of the trimer, where they generally reside at interfaces between monomers, at HA1 and HA2 subunits within a monomer, or at regions between adjacent domains within a monomer that rearrange upon acidification. Presumably, such mutations cause localized changes between domains that rearrange during fusion, and destabilize the overall structure of the neutral pH HA.

The results reported here involve mutant HAs with changes at positions that reside in the vicinity of the fusion peptide of neutral pH HA which become altered with respect to solvent accessibility and chemical environment as a result of HA precursor cleavage. Compared to cleaved HA, the HA0 precursor is unresponsive to decreases in pH based on the assays described here, suggesting that changes in environment or newly formed contacts involving these residues may be critical for the induction of fusion. A total of 21 single, double, or triple mutant HAs were analyzed and a variety of phenotypes were observed with respect to the pH of acid-induced conformational changes and membrane fusion properties.

Among the residues chosen for analysis here were HA2 Asp 109, Asp 112, and Lys 51, which are completely conserved among all HA subtypes. Individual alanine substitutions for aspartic acid residues at HA2 positions 109 and 112 were found to increase the pH of fusion by 0.3 and 0.5, respectively. These results are in agreement with previous reports regarding the mutants D1092G, D1092E, D1122G, D1122E, and D1122N, which also lead to elevated fusion pH (Daniels et al., 1985; Hoffman et al., 1997; Steinhauer, 1992, 1996). In WT HA D1092 forms hydrogen bonds to Q1052 via a water molecule and also hydrogen bonds to the main chain amide group of residue 2 of the fusion peptide. The carboxylate oxygens of WT residue D1122 form four hydrogen bonds with the amide nitrogens of HA2 residues 3, 4, 5, and 6. The five hydrogen bonds formed by these aspartic acids to the fusion peptide are present in all HAs analyzed as illustrated for H1 and H3 subtype HAs in Fig. 7. Presumably, these interactions with the fusion peptide are important for stabilizing the neutral pH structure. Our results suggest that the additive effects created by double mutation (D1092A, D1122A) destabilize HA to such an extent that its folding and transport to the cell surface is prohibited.

HA2 position 51 is situated near the middle of the shorter of the two prominent α-helices of the neutral pH HA hairpin...
structure. In all HAs, the conserved lysine side chain of HA2 51 is positioned similarly, extending into the solvent-occluded cavity towards HA2 residue 106 (Fig. 2). In the H3 group HAs, HA2 106 is usually histidine, which forms a hydrogen bond with HA2 Lys 51. In H1 group HAs, HA2 106 is either lysine or arginine, with the side chains oriented away from HA2 Lys 51. When HA2 106 is Arg, a hydrogen bond with HA2 Asp 109 of the adjacent monomer is formed (Russell et al., 2004). Our results with alanine and glutamic acid substitutions for HA2 Lys 51 indicate that these changes stabilize the neutral pH structure to some extent. Perhaps this results from the loss of repulsive interactions between this K51 and H106, side chains that may develop during acidification (see Fig. 2). This interpretation is consistent with results obtained with the HA2 106 His-to-Phe mutant, which also results in a marginal decrease in fusion pH. However, this effect is not observed with mutant HAs containing substitution of arginine or alanine at HA2 106, possibly due to other structural constraints.

Although it is not an ionizable residue, HA2 Gln 105 was chosen for analysis due to its position in the structure and based on phylogenetic considerations. Glutamine is present at this position in the H3 group HAs, and also in H9 clade HAs, whereas in other subtypes there is an acidic residue (usually glutamic acid). This is notable because for other positions in this region containing residues that segregate along group-specific lines, the H9 clade HAs fall into the H1 group (Fig. 2). In WT H3 HAs, the glutamine at HA2 position 105 forms a hydrogen bond with the amide nitrogen of HA1 residue 29 and has van der Waals contact with HA2 His 106 from an adjacent monomer, as well as forming hydrogen bonds to HA2 Asp 109 via water. Previously, a Q105R mutation was shown to elevate fusion pH by 0.3, possibly by forming a salt bridge with the aspartic acid side chain of HA2 109 and altering its interactions with the fusion peptide. In the examples reported here, the glutamic acid substitution appears to have little effect on fusion pH when taking all three assays into consideration. However, the ELISA results in particular, suggest that an alanine substitution at HA2 106 essentially had no effect on the HA fusion pH phenotype. An alanine substitution at this position caused a slight increase in fusion pH, whereas the phenylalanine mutant at HA2 106 resulted in a reduction of between 0.1 and 0.2 in the pH at which conformational changes and polykaryon formation were observed. These results are likely to be due to interactions involving residues HA2 51, 105, and 109 as discussed above.

The results of mutations at HA1 position 17 indicate that this histidine residue may play a critical role in the initiation of membrane fusion for HAs of H3 group viruses. As discussed earlier, this is one of the group-specific residues that is almost completely buried due to the relocation of the fusion peptide following cleavage. In the neutral pH HA, residue 17 forms hydrogen bonds via a water molecule to carbonyl oxygen of HA2 residues 6 and 10, and also hydrogen bonds with HA1 His 18 (Daniels et al., 1985). In the H1 group HAs, this residue is tyrosine. Previous studies with H3 subtype HAs have shown that substitution of HA1 His 17 with arginine or glutamine results in an increase of between 0.7 and 0.9 in the pH of membrane fusion (Daniels et al., 1985; Rott et al., 1984; Steinhauer et al., 1996; Wharton et al., 1986), which makes them among the least stable HAs that have been observed in infectious viruses. Our results with the H17A substitution demonstrate a similar high pH of fusion phenotype, as alanine will not be capable of forming the equivalent hydrogen bonds to the fusion peptide or the H181 carbonyl oxygen. In all probability, this loss of hydrogen bonds is responsible for the reduced stability of the mutant HA. By contrast, substitution of tyrosine for HA1 His 17 led to a reduction by 0.3 of the pH at which conformational changes and membrane fusion were detected. Modelling of tyrosine at this position and structural comparisons with the HAs of H1 group subtypes indicate that the longer side chain could allow the hydroxyl group to form hydrogen bonds directly to residues 10 and 12 of the fusion peptide (Fig. 7). Presumably, these direct bonds would be stronger than those formed via water when histidine is present at HA1 position 17, thus helping to account for the increased stability of the H17Y mutant. In addition, unlike histidine, tyrosine at this position would not have the potential to change protonation state at a pH relevant for fusion. These results suggest that for H3 group HAs, protonation of the histidine at HA1 position 17 is involved in the activation of membrane fusion.

Substitutions at HA2 position 111 resulted in elevated fusion pH, by 0.6 for T111H, 0.3 for T111V, and 0.1 for T111A, which suggests that this residue is also significant for the stability of the neutral pH structure. The identity of the residue at HA2 position 111 appears to dictate in part the group-specific
structure of a four-residue complex composed of HA2 111, HA1 18, HA1 38, and HA2 Trp 21 (Russell et al., 2004). The orientation of the side chains of HA2 Trp 21 and HA1 18 is quite different depending on whether HA2 111 is threonine, as in the H3 group HAs, or histidine, as found in the H1 group (Fig. 8). In HAs of the H3 group, the Trp 21 and His 18 side chains are aligned with one another and oriented side-on towards Thr 111, and HA1 His 17 is positioned with the face of the imidazole directed toward Thr 111. In the H1 group HAs, Trp 21 is located such that the indole side chain faces and completely buries the histidine at HA2 position 111. In these structures, the HA1 His 18 side chain orients away from HA2 His 111. The structural differences in this region could indicate group-specific mechanisms for initiating fusion, and it is possible that protonation of HA2 His 111 may play a role similar to that suggested by our results for HA1 His 17 of H3 HA as a potential trigger residue. Our initial efforts to explore this possibility by mutagenesis of HA2 His 111 in the H2 subtype HA of A/Japan/305/57 virus have been inconclusive, as all single amino acid substitution mutants examined thus far fail to express on the cell surface in the native HA conformation (data not shown).

Another indication that residues in this region of HA have evolved in coordinate fashion derives from our analysis of multiple mutants. For a number of these, it is difficult to interpret the consequences for fusion of compound changes due to structural aberrations of the expressed proteins, which suggests that the cumulative effects of two or more changes in this region decrease HA stability to an extent that the proteins are misfolded or their conformations are altered during transport to the cell surface. Among the multiple mutants that expressed on cell surfaces appropriately, the results of mutants containing H1062A and T1112A were notable. Expressed individually, each mutation caused an increase in fusion pH of 0.1, and when expressed together, the double mutant (H1062A, T1112A) resulted in an increase in the pH of conformational change of 0.4 relative to WT. However, as components of a triple mutant containing the H171Y change, the pH of structural transition was nearly indistinguishable from WT. These results are consistent with observations on the stabilizing effects of the H171Y mutation and the additive contributions of mutations within localized regions (Steinhauer et al., 1996).

Overall, the results presented here are compatible with the observation that the amino acids at positions HA1 17 and HA2 111 have segregated as a unit during evolution due to functional compatibility. Perhaps HA1 His 17 plays a role in the initial trigger for membrane fusion of H3 group HAs, and in the H1 group HAs that have tyrosine at HA1 17, the histidine at HA2 111 plays a similar role. Initial structural perturbations involving these residues may alter the chemical environment of additional residues initiating a cascade of molecular transitions involving structural intermediates (Botcher et al., 1999; Korte et al., 1999, 1997; Puru et al., 1990; Remeta et al., 2002). Secondary trigger residues or stabilizing mutations elsewhere in the HA trimer (Godley et al., 1992; Hoffman et al., 1997; Kemble et al., 1992; Steinhauer et al., 1991a) may modulate the relative stability of structural intermediates or regulate transitional “checkpoints”. A greater appreciation of the mechanisms by which the conformational changes of HA are triggered and the structural intermediates involved should aid in the further design and development of anti-influenza drugs that inhibit fusion (Bodian et al., 1993; Cianci et al., 1999; Combrink et al., 2000; Hoffman et al., 1997; Luo et al., 1997; Yu et al., 1999).

Materials and methods

Mutagenesis and expression of HAs

The HA cDNA from the H3N2 subtype virus A/Aichi/2/68 was mutated by site-directed mutagenesis using a QuickChange mutagenesis kit (Stratagene). The presence of the desired mutations and the absence of extraneous mutations were confirmed by nucleotide sequencing of entire HA coding regions. The mutant cDNAs were expressed as recombinant vaccinia viruses using the plaque-selection system (Blasco and Moss, 1995). The generated viruses were propagated on CV1 cells in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Cell surface expression

HA cell surface expression was analyzed by a trypsin cleavage assay with recombinant vaccinia-infected CV1 cells as described previously (Steinhauer et al., 1995) with a few modifications. The only difference is that blots were developed using chemiluminescence rather than radiolabeled second antibody. Following electrophoresis, blots were incubated with anti-HA rabbit polyclonal rabbit serum, washed, then incubated with a Protein A-HRP conjugate (Sigma cat# P8651). Blots were developed with the Enhanced Chemiluminescence Reagent (Amersham Pharmacia) according to the manufacturer’s instructions. Quantitative cell surface expression by ELISA was carried out using chemiluminescence rather than radiolabeled second antibody. Following electrophoresis, blots were incubated with anti-HA rabbit polyclonal rabbit serum, washed, then incubated with a Protein A-HRP conjugate (Sigma cat# P8651). Blots were developed with the Enhanced Chemiluminescence Reagent (Amersham Pharmacia) according to the manufacturer’s instructions. Quantitative cell surface expression by ELISA was carried out using 10% fetal calf serum.
out on recombinant vaccinia virus-infected HA-expressing HeLa cells using a panel of monoclonal antibodies that recognize distinct antigenic regions of wild-type HA as described (Steinhauer et al., 1991b).

**Conformational change assays**

**Trypsin susceptibility**

Recombinant vaccinia virus-infected CV1 cells were used for trypsin susceptibility assays to determine the pH of conformational changes for WT and mutant HAs. Approximately 24 h post-infection, HA-expressing infected cells were treated with trypsin (Sigma) at 5 μg/ml, 10 min, 37 °C, washed, and incubated at various pH values for 5 min at 37 °C. The medium was then returned to neutral pH and the monolayer was incubated in the presence of trypsin (5 μg/ml) for 30 min at 37 °C. The reaction was stopped by the addition of soybean trypsin inhibitor (Sigma) at a final concentration of 5 μg/ml. Subsequently, the cells were washed with PBS, and lysates were incubated at various pH values for 5 min at 37 °C. The medium formation as described previously (Li et al., 2006; Steinhauer et al., 1991b) using monoclonal antibodies HC3 and HC67. HC3 recognizes both native and low pH HA, while HC67 recognizes only the native conformation.

**Fusion assay**

The pH of membrane fusion was assayed by polykaryon formation as described previously (Li et al., 2006; Steinhauer et al., 1991b), except that the pH was reduced for 30 s.

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**References**


