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# Single residue deletions along the length of the influenza HA fusion peptide lead to inhibition of membrane fusion function

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ABSTRACT

A panel of eight single amino acid deletion mutants was generated within the first 24 residues of the fusion peptide domain of the of the hemagglutinin (HA) of A/Aichi/2/68 influenza A virus (H3N2 subtype). The mutant HAs were analyzed for folding, cell surface transport, cleavage activation, capacity to undergo acid-induced conformational changes, and membrane fusion activity. We found that the mutant ΔF24, at the C-terminal end of the fusion peptide, was expressed in a non-native conformation, whereas all other deletion mutants were transported to the cell surface and could be cleaved into HA1 and HA2 to activate membrane fusion potential. Furthermore, upon acidification these cleaved HAs were able to undergo the characteristic structural rearrangements that are required for fusion. Despite this, all mutants were inhibited for fusion activity based on two separate assays. The results indicate that the mutant fusion peptide domains associate with target membranes in a non-functional fashion, and suggest that structural features along the length of the fusion peptide are likely to be relevant for optimal membrane fusion activity.



## Introduction

All enveloped viruses encode glycoproteins that function to mediate fusion of their membranes with those of host cells during the initial stages of infection. Such viral fusion proteins (VFPs) are designed to respond to external stimuli at the appropriate time and place, to rearrange their molecular structures and initiate the fusion process. Membrane fusion can occur at the plasma membrane of host cells, often following the engagement of receptor molecules, or within subcellular compartments such as endosomes following internalization and acidification of the local environment (Harrison, 2008; Kielian and Rey, 2006; Lamb and Jardetzky, 2007; Weissenhorn et al., 2007; White et al., 2008). Often, more than one viral protein is involved, and for some viruses multiple cellular components take part in the fusion process. However, for most enveloped viruses a single membrane anchored VFP serves as the principal protagonist for drawing the membranes into proximity with one another to initiate the fusion event. For these VFPs, the structural rearrangements that trigger fusion are generally coincident with the exposure of relatively hydrophobic domains to allow for their interaction with cellular target membranes. These "fusion peptide" domains are known to occur in one of three basic forms; (i) they can be present at the N-terminus of the fusion subunit of the protein, (ii) they can reside within the polypeptide chain as single loop internal fusion peptide domains, and (iii) they can exist as internal bipartite loops.

Most of the VFPs that have been characterized appear to share some of the mechanistic features for bringing viral and cellular membranes into proximity with one another in the initial stages of the fusion process. However, VFPs are often segregated into three classes, based primarily on common structural considerations. Among these, representatives of Class I VFPs from members of the orthomyxoviridae, paramyxoviridae, and retroviridae families have been particularly well characterized. For these VFPs, polypeptide precursors associate to form trimers that are cleaved to generate N-terminal fusion peptides on their membrane-anchored subunit. During the fusion process, these membrane-anchored subunits undergo conformational changes to generate highly stable helical rod structures. As a consequence, the N-terminal fusion peptides are relocated to the same end of the molecule as the C-proximal viral membrane anchor domain. The molecular rearrangements and end-state structures for Class I VFPs are consistent with a model for membrane fusion, in which N-terminal fusion peptides are directed to interact with cellular target membranes, and formation of helical rod structures bring the viral and cellular membranes into proximity to one another as a prelude to the fusion process.

The influenza A virus hemagglutinin glycoprotein (HA) serves as the prototype for the Class I VFPs. It is synthesized as polypeptide chains of approximately 550 amino acids that associate non-covalently as homotrimers. The precursor form of the trimer (HA0)

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requires proteolytic cleavage of each monomer into the disulfide-linked subunits HA1 and HA2 in order to activate membrane fusion potential and virus infectivity (Appleyard and Maber, 1974; Klenk et al., 1975; Lazarowitz and Choppin, 1975). Cleavage of HA0 not only liberates the HA2 N-terminal fusion peptide domain, but allows the HA to assume a neutral pH conformation that can subsequently be triggered by acidification to undergo the conformational changes required for membrane fusion (Bizebard et al., 1995; Bullough et al., 1994; Chen et al., 1999).

Although the fusion peptides of all Class I VFPs feature several large hydrophobic amino acids and include a number of glycine residues interspersed along their length, there is no direct sequence homology outside of the individual virus families. By contrast, the HA fusion peptide domains of all16 HA subtypes of influenza A viruses, as well as those of influenza B viruses, are highly homologous. This is particularly evident within the N-terminal 11 residues, and at positions with large hydrophobic or glycine residues (Fig. 1A). The reason for such conservation is likely due to constraints on the folding of the precursor HA0 structure, the capacity of this structure to be cleaved into a neutral pH structure that is responsive to acidification, and the requirement that fusion peptides be capable of adopting functional structures while interacting with target membranes to initiate the process of membrane fusion.

A number of studies on expressed proteins and mutant influenza viruses have focused on the membrane fusion properties of mutant HAs with changes in the fusion peptide domain (Cross et al., 2001; Daniels et al., 1985; Gething et al., 1986; Korte et al., 2001; Lai and Tamm, 2007; Lin et al., 1997; Nobusawa et al., 1995; Qiao et al., 1999; Steinhauer et al., 1995; Yewdell et al., 1993). Among the implications derived from these studies, the N-terminal glycine has been demonstrated as particularly important for fusion activity, and large hydrophobic residues at positions 2, 3, 6, 9, and 10 are desirable for optimal function. The conserved tryptophan at HA2 position 14 also appears to play a fundamental role for fusion. The glycine at position 4 has been shown to tolerate changes in functional HAs, but the glycine at position 8 may be more critical, and numerous observations suggest that the spacing of glycine residues in fusion peptides may be important for fusion peptides to adopt functional structures.

The actual length of fusion peptide domains has not been addressed in great detail, particularly as components of intact HA molecules. Experiments on mutants with a deletion of either the N-terminal glycine or the leucine at HA2 position 2 were shown to be non-functional, using assays with synthetic peptide analogs or full length expressed HAs (Steinhauer et al., 1995; Wharton et al., 1988). Further evidence for length constraints in the terminal region of the influenza fusion peptide derive from studies on cleavage activation mutants selected for growth in the presence of the protease thermolysin (Orlich and Rott, 1994). This protease cleaves HA0 between the residues that normally constitute HA2 Gly1 and Leu2, which generates an HA2 subunit with leucine at the N-terminus and a fusion peptide that is truncated by one residue. However, mutants selected for growth in the presence of thermolysin were found to contain single residue insertions just downstream of the N-terminal leucine, which functioned to restore authentic fusion peptide length.

For the present study, we extended previous analyses on the length requirements for functional influenza HA fusion peptide sequences using expressed HAs with single residue deletions that span this domain. We found that with one exception, the mutants fold into native HAs that were expressed on cell surfaces, cleaved into HA1 and HA2, and were able to undergo the acid-induced conformational changes requisite for fusion. However, all mutants were debilitated for membrane fusion function, suggesting either an overall length requirement for these fusion peptide sequences, a requirement for particular structural elements along the length of this domain, or

**A. Fusion peptide sequences of representatives for each HA subtype**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H1 | G | L | F | G | A | I | A | G | F | I | E | G | G | W | T | G | M | I | D | G | W | Y | G | Y | H |
| H2 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Q | – | – | V | – | – | – | – | – | Y | H |
| H3 | – | – | – | – | – | – | – | – | – | – | – | N | – | – | E | – | – | – | – | – | – | – | – | F | R |
| H4 | – | – | – | – | – | – | – | – | – | – | – | N | – | – | Q | – | L | – | – | – | – | – | – | F | R |
| H5 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Q | – | – | V | – | – | – | – | – | Y | H |
| H6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Y | H |
| H7 | – | – | – | – | – | – | – | – | – | – | – | N | – | – | E | – | L | V | – | – | – | – | – | F | R |
| H8 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | S | – | – | – | – | – | – | – | – | F | H |
| H9 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | P | – | L | V | A | – | – | – | – | F | Q |
| H10 | – | – | – | – | – | – | – | – | – | – | – | N | – | – | E | – | – | V | – | – | – | – | – | F | R |
| H11 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | P | – | L | – | N | – | – | – | – | F | Q |
| H12 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | P | – | L | V | A | – | – | – | – | F | Q |
| H13 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | P | – | L | – | N | – | – | – | – | F | Q |
| H14 | – | – | – | – | – | – | – | – | – | – | – | N | – | – | Q | – | L | – | – | – | – | – | – | F | R |
| H15 | – | – | – | – | – | – | – | – | – | – | – | N | – | – | E | – | L | – | – | – | – | – | – | F | R |
| H16 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | P | – | L | – | N | – | – | – | – | F | Q |

**B. H3 subtype fusion peptide deletion mutants for this study**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | G | L | F | G | A | I | A | G | F | I | E | N | G | W | E | G | M | I | D | G | W | Y | G | F | R |
| ΔL2 | – | Δ | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ΔG4 | – | – | – | Δ | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ΔI6 | – | – | – | – | – | Δ | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ΔF9 | – | – | – | – | – | – | – | – | Δ | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ΔN12 | – | – | – | – | – | – | – | – | – | – | – | Δ | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ΔE15 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Δ | – | – | – | – | – | – | – | – | – | – |
| ΔD19 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Δ | – | – | – | – | – | – |
| ΔF24 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Δ | – |

**Fig. 1.** A) HA Fusion peptide sequences from representatives of each of the 16 HA subtypes. H1: A/PR/8/34, H2: A/Japan/305/57, H3: A/Aichi/2/68, H4: A/Duck/Czechoslovakia/56, H5: A/Chick/Pennsylvania/1370/83, H6: A/Shearwater/Australia/1/72, H7: A/FPV/Rostock/34, H8: A/Turkey/Ontario/6118/68, H9: A/Turkey/Wisconsin/66, H10: A/Chicken/Germany/N/49, H11: A/Duck/England/56, H12: A/Duck/Alberta/60/76, H13: A/Gull/Maryland/704/77, H14: A/Mallard/Astrakhan/263/1982, H15: A/Duck/Australia/341/1983, H16: A/Black-Headed Gull/Sweden/2/99. B) Nomenclature and fusion peptide sequences of the HA deletion mutants addressed in this study. The symbol Δ denotes the deletion of the amino acid residue at this position.