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Hypothesis

An architecture for the fusion site of Influenza hemagglutinin

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The recent finding that more than one Influenza hemagglutinin (HA) is required at the fusion site for HA-expressing fibroblasts [1], together with the crystal structure of HA at neutral pH [2], provide the basic elements of a plausible model for this fusion site. Within an aggregate of HA trimers at low pH, we propose fusion intermediates which are based upon a minimal alteration to the known neutral pH structure of HA and which should have reasonable activation energies. This is the first model of a glycoprotein-mediated fusion site which explicitly accounts for the disposition of the lipids within these intermediates. While the fusion site created by HA will not be the same as that of eukaryotic fusion complexes [3], general characteristics could be shared.

Influenza hemagglutinin; Membrane fusion; Fusion protein; Liposome; Lipid-protein interaction; Envelope glycoprotein

1. INTRODUCTION

Ten years ago, the X-ray crystallographic structure of the bromelain-released, soluble ectodomain of the (A/Aichi/2/68) Influenza envelope glycoprotein hemagglutinin (BHA) was solved [2]. Soon after, BHA and the intact hemagglutinin (HA) were shown to undergo similar conformational changes at the pH associated with fusion between the viral envelope and a target membrane [4]. As a mature protein, HA consists of two polypeptide chains linked by a single disulfide bond. HA1 contains the sialic acid binding site of HA [5]. The N-terminal region of the HA2 subunit is a highly conserved apolar sequence which is exposed at low pH [6] and which can control the fusion function of HA [7]. These 20 or so amino acids are referred to as the fusion peptide sequence of HA [7,8].

While many molecular details of the low pH-induced conformational changes of HA are known [6–15], it is not known how HA actually induces fusion. A crucial parameter for elucidating the architecture of the fusion site, i.e. the arrangement of the fusion proteins and the lipid bilayers, is the number of HAs at the fusion site. To determine this number, it is necessary to measure fusion efficiency as a function of HA surface density [16].

Ellens et al. [1] studied this problem using two NIH 3T3 fibroblast cell lines which stably express the HA from the Japan strain of Influenza virus at different cell surface densities. Fluorescence recovery after photobleaching showed that the HAs on both cell lines had the same lateral diffusion coefficients and the same mobile fractions, which indicates that there was no significant difference between the HAs expressed on the plasma membranes of both cell lines. Fusion efficiency was measured using glycophorin bearing liposomes (diameter ~ 0.5 μm). These liposomes were shown to bind to the HA-expressing cells specifically through HA-glycophorin interactions. Surprisingly, it was found that the low pH induced fusion between the cells and bound liposomes was mediated by HAs not bound to glycophorin. Thus, while each HA has a binding function and a fusion function, these functions are not carried out by the same HA. The other significant finding was rigorous proof that more than one HA trimer is required to induce fusion. This had been speculated previously [11] and a few studies have aimed at deducing this minimal fusion unit, using indirect methods for altering the surface density of 'fusogenic' HA on Influenza virus or HA-expressing cells [17,18].

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2. PROPOSAL

These findings led us to reconsider the mechanism of HA-induced fusion. In particular, we sought to account for the disposition of the bilayer lipids within fusion intermediates which qualitatively minimize the activation energy of the process. The model which we propose has three separate elements. Firstly, we propose that the HAs at the fusion site form a 'collar' and that fusion of

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the membranes occurs within this collar. Secondly, we propose that the role of the fusion peptide is to dehydrate the interior of this collar, which induces a wetting of the HAs by the lipids in the membranes. This is the initial step of membrane destabilization. Thirdly, we propose that the fusion peptide accomplishes this dehydration and wetting not by inserting into either membrane, but rather by residing between the apposed membranes, where its hydrophobic potential draws the lipids from both bilayers into the fusion site. We will describe this model first and then discuss how it differs from previous descriptions of HA-induced fusion.

The essential difference between an isolated HA trimer and a multimer of HA trimers is the space within the multimer, e.g. where the HAs could form a 'collar'. This space is a natural candidate for the fusion site. Since the assay for fusion used in Ellens et al. [1] was the transmission of a soluble, liposome-encapsulated, 30 kDa plant toxin into the cells, it was evident that proper fusion through a pore had been achieved. In Fig. 1, a top-view of a lipid-lined fusion pore surrounding an aqueous channel is shown. Rigorously, we only know that more than one HA is at the fusion site, but it does not seem likely from scale modelling that only two HAs would be sufficient. Thus, we show three HAs at this fusion site, although this choice is arbitrary. The amphipathic N-terminus of HA2 is shown as an α-helix at the oil/water interface of the lipid-lined pore and as a random coil in the aqueous space [8,14,15]. The diameter of the pore shown is about 4 nm, which is the estimated initial pore size for the fusion of the HA-expressing cells with erythrocytes [19,20].

How does the aggregation of HA trimers create the fusion site? We propose that at low pH, the close approach of HA trimers with exposed amphipathic fusion peptides dehydrates the intermembrane space within the collar, which promotes the wetting of the inner surface of the HAs in the collar. This is shown by the formation of a localized lipid intermembrane intermediate (Fig. 2A). HAs bound to glycophorin are not shown since

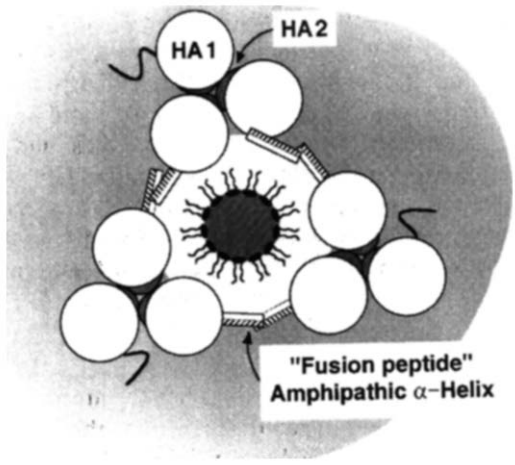

Fig. 1. Top view of the proposed lipid-lined fusion pore, with an aqueous channel, created by three HA trimers at low pH. The exposed fusion peptides are α-helices at the interfaces between the acyl chains and the medium, and they are random coils when exposed to just the aqueous medium [8,14,15].

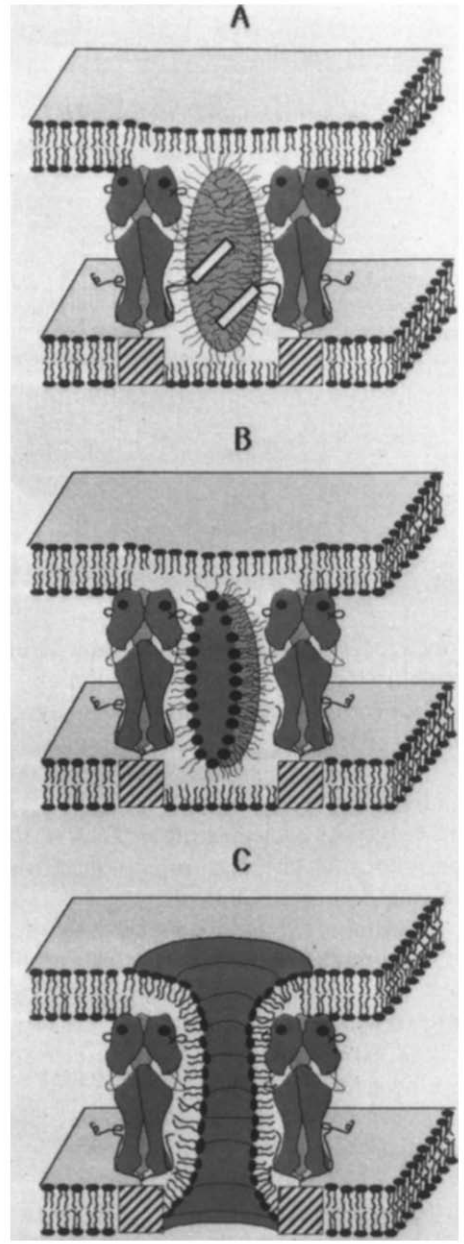

Fig. 2. Proposed structures of the HA fusion intermediates. (A) Close approach of two or more HA trimers, with the exposed amphipathic fusion peptides, dehydrates the intermembrane space, which permits the formation of a closed intermembrane intermediate, like an inverted micelle. The HA1 globular headgroups for the Japan strain do not dissociate [25], as discussed in the text. The packing of the lipids near the transmembrane domains and the tops of the HA1 headgroups are not specified. (B) A cut-away view of the intermediate, showing the aqueous core. (C) The unstable intermembrane intermediate shown in part (A) will either revert to two apposed membranes or it will break perpendicular to the membranes and form the lipid-lined fusion pore shown here.

they are not directly involved in fusion [1]. The cut-away view in Fig. 2B shows the aqueous core of the intermediate. This intermediate is unstable and either will revert to the apposed membranes, or it will break perpendicular to the membranes and form an interlamellar attachment. Fig. 2C shows a cut-away view of this interlamellar attachment, i.e. the lipid-lined fusion pore. Fig. 1 serves as a top-view for the structures shown in Fig. 2. In pure lipid systems, the formation of intermembrane intermediates and interlamellar attachment sites (similar to those shown here), as well as their mediation of liposome fusion, have been very well characterized [21–23]. The conductance and capacitance flickering observed by Spruce et al. [20] prior to the complete fusion of HA-expressing cells with erythrocytes would be explained by breakage of the pore and reformation of the apposed membranes. After pore formation, the HAs will diffuse apart, thereby widening the pore and completing the fusion event. Knoll et al. [24] have shown freeze-fracture images from Influenza virus infected cells fusing with liposomes which are consistent with the structures shown in Fig. 2C (see also [23]).

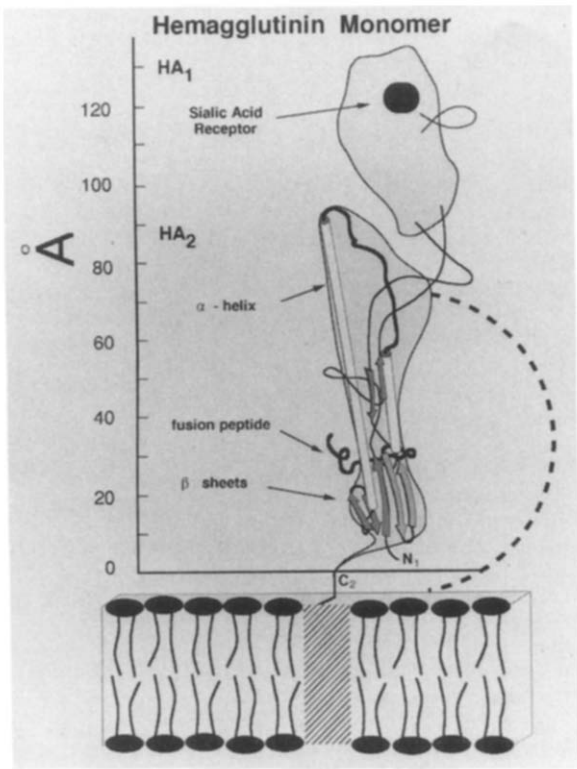

Fig. 3. The secondary structure of the HA monomer is shown at neutral pH (adapted from Wilson et al. [2] and Ruigrok et al. [9]). The HA1 globular headgroups contain the sialic acid binding site for HA, shown by the dark spot on HA1, which is the site of glycophorin binding. The fibrous HA2 bodies contain the fusion peptide at the N-terminus, which is tucked into the center of the trimer at neutral pH and exposed at low pH [2,6]. Following the low pH treatment, the fusion peptide is exposed and we propose that its motion is restricted to within the area demarked by the dashed line, due to constraints in secondary and tertiary structure. Only the monomer is being shown here for clarity, but it is important to recall that the HA trimer remains intact at low pH [11].

Dissociation of the globular HA1 subunits is known to occur following low pH treatment with many Influenza virus strains, including A/Aichi/2/68 [6]. Interestingly, for the Japan strain used in Ellens et al. [1], it has been reported that this dissociation does not occur and does not represent a necessary step in fusion [25]. Thus we show the HA1s in an undissociated conformation during fusion. Our model does not depend upon whether dissociation occurs. It is only a question of whether the distance between the membranes at the fusion site is 9 or 13 nm, these being the heights of the HA2 trimer and the intact HA trimer, respectively. This distance could be shortened by having the HAs 'lie down' onto the membrane surface, but this entails postulating additional conformational changes to HA.

In Fig. 3, we show why it is reasonable to speculate that the fusion peptide of HA acts between the apposed membranes, rather than inserting into either one. The secondary structure of the HA monomer (for A/Aichi/2/68) is shown at neutral pH (adapted from Wilson et al. [2] and Ruigrok et al. [9]). About 30 amino acids in from the N-terminus, the polypeptide sequence of HA2 forms into two strands of a five stranded β-sheet, where the other three strands derive from distal portions of the HA monomer [2]. After these two strands, the polypeptide chain directly forms into a small α-helix, which is conjugated with the main α-helix of the fibrous stem of HA2 [3]. So long as these parts of the secondary and tertiary structure remain intact by the dashed line. The ~40 A radius of this hemisphere is the length of the N-terminus (as an α-helix) out to the beginning of the first strand of the β-sheet. Two points must be recalled. Low pH treatment does not cause the trimer to dissociate [11] and it does not change the overall α-helix or β-sheet content of HA [10]. While the morphology of HA (X-31) on isolated virus eventually changes at low pH [9–11], these changes need not reflect the situation of HA at the fusion site with an apposed membrane. Thus, if HA remains 'upright' during fusion, then the site of action of the fusion peptides might well be between the apposed membranes, where their hydrophobic potential draws the lipids from both membranes into the middle of the fusion site.

3. DISCUSSION

This proposal differs from the traditional view that fusion is initiated by the insertion of the fusion peptide into either the target membrane [7,8,11–14,26,27] or the viral membrane [9,10,13]. As shown in Fig. 3, additional conformational changes to HA need to be postulated in order to permit the fusion peptide to reach

either of the two membranes. In our model, only the exposure of the fusion peptide is required. In this sense, our model proposes a minimal change to the known neutral pH structure of HA in order to explain its fusion activity.

Recent studies by Brunner and colleagues are especially interesting. They have used a lipid bound photoactivatable carbene probe to show that the fusion peptide sequence of BHA binds *only* to the outer monolayer of liposomes and does so with only one side of the α-helix embedded into that monolayer [14,15]. This is reasonable, since an apolar sequence exposed on a soluble protein will seek a hydrophobic environment, i.e. either a lipid bilayer or through self-aggregation [10,14]. However, this does not necessarily imply that the fusion peptide of the intact HA in a membrane will also insert into an apposing membrane, since HA does not have the same rotational freedom as BHA. On the other hand, this is precisely the depth of interaction and the configuration required by our model (Fig. 2) for the fusion peptide on HA, i.e. where one side of the α-helix faces water and the other side faces the acyl chains of the lipids.

In fact, it is not obvious how insertion of a few amphipathic α-helices into the outer monolayers of either or both apposed membranes generates the required destabilization. In pure lipid systems, these bilayers are stable down to 5 Å separation [28]. Peptides homologous to those found on the N-terminus of HA2 do destabilize small sonicated liposomes (SUV) at low pH [8,13]. It is known that SUV are typically more unstable than larger liposomes [29]. Even so, destabilization required very high peptide-to-lipid ratios, where lysis is followed by lipid mixing. In fact, the action of these peptides at low pH is rather similar to the action of detergents like cholate or octylglucoside [30]. An analogous 20 amino acid peptide, which becomes α-helical at low pH, has been estimated to produce $\sim$0.5–1 nm pores in liposomes, with about 8–12 peptides being required to form each pore [31]. This behaviour is not consistent with the fusion induced by HA. In our view, the position of the fusion peptide within the fusion site is at least as important as its hydrophobicity and secondary structure.

Several different experimental approaches will be required to elucidate the architecture of the fusion site. Our model proposes that the lipids wet the proteins at the fusion site, rather than having the lipid bilayers spontaneously fuse following insertion of the fusion peptide. In principle, experiments using lipid bound photoactivatable carbene probes, such as those performed on BHA [14,15] and Sendai virus [32], would be useful, in that the wetted proteins would be labelled at both the fusion peptide and at other sites along HA. However, these experiments will not be simple to perform or to analyze. Firstly, it will be essential to correlate the labeling kinetics with the fusion kinetics, since postfusion conformational changes of HA may well occur. Secondly, it is essential to recall that while there are many HAs within the area of membrane contact, very few will be involved in fusion. Those not involved in fusion may well be labelled by the probe.

To conclude, the sequence of fusion intermediates shown in Fig. 2 begins the task of minimizing the thermodynamic costs of breaking open bilayers in order to achieve fusion. The exposure of acyl chains to water can be alleviated by proposing more extensive conformational changes to HA than we have done here. This becomes reasonable once more is known about the low pH, fusogenic conformation of HA. Using the crystal structure of HA, we can examine how the surface of this protein, as an element of the HA aggregate, might facilitate the flow of lipids between the bilayers. This knowledge will be invaluable in elucidating how other proteins induce fusion, since the 'collar' would appear to be an excellent candidate for a general architecture of fusion sites, even for eukaryotic systems [4].

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