

Membrane Fusion Mediated by Coiled Coils: A Hypothesis

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ABSTRACT A molecular model of the low-pH-induced membrane fusion by influenza hemagglutinin (HA) is proposed based upon the hypothesis that the conformational change to the extended coiled coil creates a high-energy hydrophobic membrane defect in the viral envelope or HA expressing cell. It is known that 1) an aggregate of at least eight HAs is required at the fusion site, yet only two or three of these HAs need to undergo the “essential” conformational change for the first fusion pore to form (Bentz, J. 2000. *Biophys. J.* 78:000–000); 2) the formation of the first fusion pore signifies a stage of restricted lipid flow into the nascent fusion site; and 3) some HAs can partially insert their fusion peptides into their own viral envelopes at low pH. This suggests that the committed step for HA-mediated fusion begins with a tightly packed aggregate of HAs whose fusion peptides are inserted into their own viral envelope, which causes restricted lateral lipid flow within the HA aggregate. The transition of two or three HAs in the center of the aggregate to the extended coiled coil extracts the fusion peptide and creates a hydrophobic defect in the outer monolayer of the virion, which is stabilized by the closely packed HAs. These HAs are inhibited from diffusing away from the site to admit lateral lipid flow, in part because that would initially increase the surface area of hydrophobic exposure. The other obvious pathway to heal this hydrophobic defect, or some descendent, is recruitment of lipids from the outer monolayer of the apposed target membrane, i.e., fusion. Other viral fusion proteins and the SNARE fusion protein complex appear to fit within this hypothesis.

INTRODUCTION

Membrane fusion is a crucial event in a multitude of biological processes, and understanding the molecular mechanism is a central goal of biology. The ectodomain of the membrane fusion glycoprotein hemagglutinin (HA) of influenza virus, which requires low pH to initiate fusion, was the first membrane fusion protein whose crystal structure was solved (Wilson et al., 1981), and it remains the prototypical fusion protein (Skehel and Wiley, 1998; Sutton et al., 1998; Baker et al., 1999). The key difference between the “native” structure of HA and the low-pH structure is the formation of an extended coiled coil starting from the N-terminus of the native coiled coil and a helix-turn occurring within the C-terminal end of the native coiled coil, near the transmembrane domain (Carr and Kim, 1993; Bullough et al., 1994; Chen et al., 1995). Recently, ectodomain core complexes of other membrane fusion proteins have shown remarkably similar equilibrium crystal structures with re-

spect to this coiled coil motif (Chan et al., 1997; Fass et al., 1996; Weissenhorn et al., 1997, 1998; Caffrey et al., 1998; Sutton et al., 1998; Skehel and Wiley, 1998; Baker et al., 1999; Singh et al., 1999).

This has led to speculation that these proteins share a common molecular mechanism for initiating membrane fusion. The belief is that the formation of extensive coiled coils can release adequate free energy to overcome the barriers to membrane fusion. Curiously, the formation of the extended coiled coil, which is the conformational change of HA likely to release the most energy, has been speculated solely to bring the HA2 N-terminus, aka the fusion peptide, to the target membrane, a process that one would think to be nearly spontaneous. However, the conformational change speculated to form the high-energy membrane defect is the helix-turn near the transmembrane domain. This is essentially a rearrangement of hydrophobic interactions within preexisting coiled coil domains and would release much less energy than the formation of the extended coiled coil (Carr and Kim, 1993; Bullough et al., 1994; Hernandez et al., 1996; Weissenhorn et al., 1997, 1998; Skehel and Wiley, 1998). These assignments do not appear to optimally utilize the free energy released by the conformational changes.

Correlating HA conformational changes with its fusion mechanism requires a rigorous kinetic analysis of both. A first step for the fusion mechanism has been accomplished in Bentz (2000) where the data of Melikyan et al. (1995a) were analyzed for the time required for the first fusion pore to form (i.e., the first electrical conductivity event) across a planar bilayer induced by bound HA expressing cells. These kinetic data showed that the first fusion pore formed from an aggregate of at least eight HAs, of which *only* two or three had to undergo the “essential” HA conformational change, which has a rather slow average halftime of 10^4 s.

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Abbreviations used: HA, hemagglutinin trimer; BHA, bromelain-solubilized hemagglutinin ectodomain; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; Env, the fusion protein of HIV; GD1a, ganglioside GD1a; HIV, human immunodeficiency virus; LPC, lyso phosphatidylcholine; SNARE, family of membrane proteins involved in eukaryotic intracellular trafficking and fusion of vesicles (containing the v-snare) with intracellular target membranes (containing the conjugate t-snares); TBHA2, a proteolytic fragment of the low-pH form of BHA, comprising residues 38–175 of HA2 and 1–27 of HA1.

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Here, the results of this kinetic analysis are coupled to the proposal that the formation of the extended coiled coil of HA is the defect forming conformational change. Mere close apposition of lipid bilayers (≥ 1 nm) does not lead to “spontaneous” fusion. A hydrophobic and/or curvature defect in the membranes is required. Since only two or three HAs need to transform slowly to initiate fusion, this strongly suggests that the first destabilization is a hydrophobic defect, since creation of a bending defect should require the concerted effort of many more HAs (Kozlov and Chernomordik, 1998). We begin with the model for HA-mediated fusion and other fusion proteins will be discussed later.

HA conformations and fusion intermediates

In the physiological course of infection, virus bound to the cell surface is endocytosed and exposed to low pH, which induces two or three subsequent conformations in HA, which are depicted in Fig. 1. The native structure of HA (Fig. 1, conformation 1) is based upon the crystal structure of the bromelain-released hemagglutinin ectodomain, BHA (Wilson et al., 1981). HA is a homotrimer and each monomer is composed of two polypeptide segments, designated HA1 and HA2. The HA1 segments form sialic acid binding sites, which mediate initial HA attachment to the host cell surface. The HA2 segments form the membrane-spanning anchor, the assembly domain of the homotrimeric structure, and its amino-terminal region is required in the fusion reaction (Gething et al., 1986).

Upon acidification, exposure of the amino terminus of HA2, known as the fusion peptide, occurs (Fig. 1, conformation 2). This change is rapid compared to fusion and is required to promote fusion between the viral envelope and the target membrane (Skehel et al., 1982; White and Wilson, 1987; Stegmann et al., 1990; Stegmann and Helenius, 1993; Godley et al., 1992; Pak et al., 1994).

While there has been a long literature proposing that the exposed N-terminal of HA next inserts into the target membrane to start fusion (reviewed in Durell et al., 1997), it is more accurate to say that HAs can have their fusion peptides either suspended between the membranes or embedded in the target bilayer or in their own bilayer (Bentz et al., 1990; Gaudin et al., 1995; Shangguan et al., 1998; Kozlov and Chernomordik, 1998). The fraction in each state probably depends upon time and the real question is: Which state or which sequence of states is on the “fusion pathway”? Kozlov and Chernomordik (1998) and Bentz (2000) argue that those HAs whose fusion peptides embed into the viral/HA expressing cell envelope are on the fusion pathway. Reaching the target membrane represents a later step in the process. Thus, the fusion peptide is shown here to be embedded in the viral envelope and this is the population we shall follow.

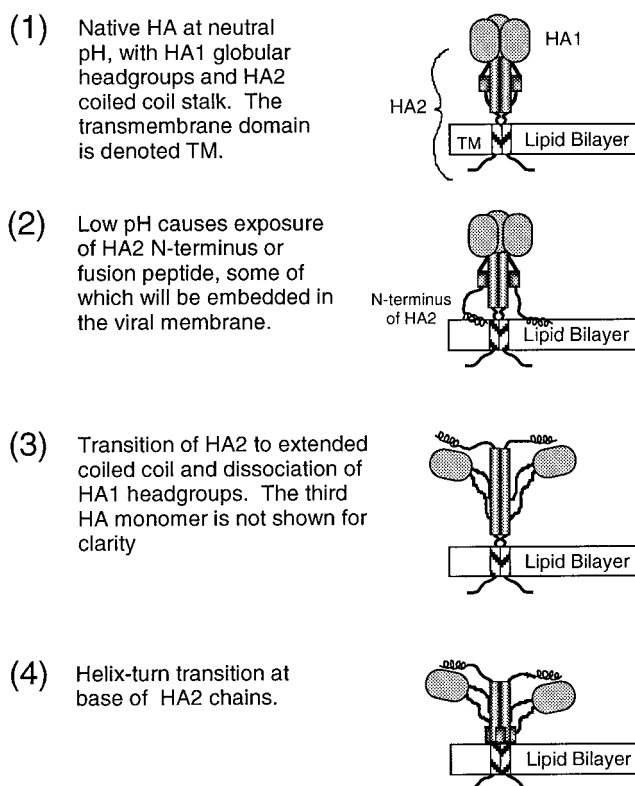


FIGURE 1 The conformations of HA. (1) This is the native conformation of BHA, adapted from Wilson et al. (1981). The globular HA1 headgroups sit on top of the spike-like HA2. Regions of α -helix and coiled coil are shown as cylinders. The bottom aggregate of α -helices is the transmembrane domain, striped and denoted TM. (2) Low pH releases the HA2 N-terminus, also known as the fusion peptide, which is shown here as embedded in the viral envelope, although other states are also possible. (3) The transition to the extended coiled coil by HA2 causes dissociation of the HA1 headgroups. (4) The helix-turn transition at the base of the original coiled coil of HA2 found in the crystal structure of TBHA2 (Bullough et al., 1994) is shown, with the hydrophobic faces aimed into the pockets vacated by the fusion peptides. Whether this transition happens with membrane bound HA is not yet known.

The second conformational change leads to the formation of the extended coiled coil of HA2 (Fig. 1, conformation 3), which was predicted by Carr and Kim (1993), proven for the crystallographic structure of a fragment of BHA (TBHA2, residues 38–175 of HA2 and 1–27 of HA1 held together by the disulfide bond) by Bullough et al. (1994) and morphologically observed on the intact virus by Shangguan et al. (1998). In addition, Qiao et al. (1998) showed that site-directed point mutations predicted to inhibit the formation of the extended coiled coil did inhibit the fusion of erythrocytes to HA expressing cells. This conformational change relocates the N-terminus of HA2 ~ 10 nm toward the target membrane, creates new stretches of coiled coil structure, and should release the largest free energy of all the HA conformational changes.

The crystal structure of TBHA2 (Bullough et al., 1994) also shows that the C-terminus of the coiled coil of BHA

has flipped up to a helix-turn between residues 106 and 112 of HA2 and an antiparallel α -helical annulus from residues 113–129 of HA2, i.e., at the base of the extended coiled coil (see Fig. 1, conformation 4). Basically, the C-termini of the native coiled coil flip up so that their hydrophobic faces can now fill the hydrophobic pockets evacuated by the fusion peptides, thereby forming the annular helices around the C-terminus of the low-pH coiled coil. This helix-turn transition, as yet unproven for membrane-bound HA, has been proposed as a means of bringing the membranes together (Bullough et al., 1994; Chen et al., 1995; Hernandez et al., 1996; Carr et al., 1997; Weissenhorn et al., 1997, 1998; Skehel and Wiley, 1998). However, since it only rearranges preexisting hydrophobic domains of coiled coil to cover the hydrophobic pocket evacuated by the fusion peptide, while losing some hydrophobic contacts, it should not release nearly as much free energy as the newly formed extended coiled coil. The C-terminal helical extension of TBHA2 found in Chen et al. (1999) was not observed using the original expression system (Bullough et al., 1994), which suggests that it is due to a still weaker interaction than the core helix-turn. Whether this extension reflects the *in vivo* structure is not known. Obviously, even assuming that the helix-turn occurs on the viral HA, the order of the changes to extended coiled coil and to the helix-turn transition isn't known.

The current problem in HA-mediated fusion is to correlate these well-known "individual" low-pH conformational changes of HA fragments with the "communal" intermediates of fusion. Most proposed HA-mediated fusion mechanisms are composed of four distinct intermediates, subsequent to close apposition of the membranes and the low pH-induced exposure of the HA2 N-terminus (Bentz, 1992, 2000; Blumenthal et al., 1996; Chernomordik et al., 1998). Currently, these intermediates are:

1. Aggregates of HA, which are either preformed or form rapidly subsequent to acidification. ω denotes the minimal aggregate size required to sustain the formation of the next step;
2. The first fusion pore defined by the first conductivity (2–5 nS) across the membranes. Additional flickering pores follow that lead to the formation of a terminally open pore;
3. The lipidic channel, which is monitored by lipid dye transfer between membranes;
4. The fusion site, which is monitored by aqueous contents mixing (e.g., fluorophors) and the stable joining of the two membranes and complete aqueous contents mixing.

It is generally accepted that an aggregate of HAs is required to form the fusion site (Ellens et al., 1990; Bentz et al., 1990; Bentz, 1992, 2000; Blumenthal et al., 1996; Danieli et al., 1996). The analysis of the data of Melikyan et al. (1995a) in Bentz (2000) showed that rapid HA aggregation followed by a slow "essential" conformational change

of the HAs within the aggregate fitted the data far better than the opposite assumption of fusion, which is rate-limited by slow HA aggregation. The mechanism of rapid HA aggregation could be adhesion of the fusion peptides within the aqueous space (Ruigrok et al., 1988), and/or membrane curvature minimization due to fusion peptides embedded in their own (viral or HA expressing cell) membrane (Kozlov and Chernomordik, 1998), which is consistent with the hypothesis made here, and/or some other interaction.

Since there is conductivity across the membranes at the first fusion pore, in the absence of lipid or aqueous dye mixing, this implies that initially there is restricted lipid flow into the fusion site from the viral or HA expressing cell bilayer (Tse et al., 1993; Zimmerberg et al., 1994; Chernomordik et al., 1998). Furthermore, the step from first fusion pore to lipid channel appears to be the committed step to fusion, since once sustained flickering occurs complete fusion usually follows. Chernomordik et al. (1998) also discuss other side reactions from the first fusion pore that do not lead to fusion, further implicating the transition from first fusion pore to lipid channel as the committed step of fusion.

Kemble et al. (1994) and Melikyan et al. (1995b, 1997) found that GPI-linked HAs on cells only induce outer monolayer mixing, thus showing the necessity of the full transmembrane domain for achieving the committed step for fusion. Other viral fusion proteins appear to require flexibility in their transmembrane domains for activity (Cleverley and Lenard, 1998). Chernomordik et al. (1998) suggested that the function of the HA transmembrane domains is to restrict the flow of lipids into the fusion site by a close approach due to aggregation of the transmembrane domains. The GPI-linked HAs may fail to promote fusion because they fail to restrict this flow. While the evidence of the state of restricted lipid flow is clear (Tse et al., 1993; Zimmerberg et al., 1994; Chernomordik et al., 1998), the real question is: Why is restricted lipid flow important for fusion?

The committed step of HA-mediated fusion

Following the paradigm for enzymology, the key to understanding the committed step of any multistep process is to understand how the "enzyme" is complementary to the "transition state." For HA-mediated fusion, we ask what kind of "transition" state can be stabilized by restricting the flow of lipids from the viral bilayer into the fusion site. The highest energy state in fusion will be the initial destabilization or disruption of the apposed monolayers, which is resisted by the hydrophobic effect and bilayer curvature effects (Siegel, 1993a, b; Chernomordik et al., 1995). The only substantial available energy to facilitate this process, aside from thermal energy, is the conformational changes of HA (see Fig. 1, conformations 3 and 4).

Fig. 2 is the hypothesis for fusion mediated by coiled coils. Fig. 2 *A* shows the aggregate of HAs with fusion peptides embedded in the viral envelope, which is the starting point of the model of Kozlov and Chernomordik (1998) for HA aggregation and fusion. While other HAs may have their fusion peptides in the aqueous media or in the target membrane, here we will follow the path of those HAs that begin with their fusion peptides embedded in their own envelope. These HAs seem most likely to blaze the fusion pathway.

The conformational change to the extended coiled coil is assumed to occur next (Skehel and Wiley, 1998; Baker et al., 1999), as depicted in Fig. 2 *B*. This must extract the fusion peptides from the outer monolayer of the viral envelope and send them toward the target membrane. For those HAs in isolation or in aggregates not in the restricted flow state, the evacuated space in the viral outer monolayer will be quickly filled by lipid diffusion and little or nothing significant will happen on the viral envelope. Presumably these fusion peptides would embed in the target membrane, which would explain the hydrophobic binding of virions to target membranes, e.g., liposomes, which occurs before fusion (Stegmann and Helenius, 1993; Brunner and Tsuredome, 1993; Chernomordik et al., 1998; Bentz, 2000). However, for those fusion peptides embedded within the site of restricted lipid flow, the evacuated space cannot be refilled instantly since the aggregated HA transmembrane domains and the remaining embedded fusion peptides would block the flow. Thus, a hydrophobic defect is created. It is important to note that because the depiction shown in Fig. 2 *B* is to scale, albeit crudely, it shows that the initial hydrophobic defect must expose both acyl chains and HA transmembrane domains to the water. Thus, the surface of the defect is a mixture of a hydrophobic lipid and protein. This will become very important as this hypothesis evolves.

The first obvious question is whether there is enough energy released in the formation of the extended coiled coil to extract the fusion peptide and produce this hydrophobic defect. The answer is yes, within the limits of our current knowledge. A slightly more sophisticated answer is that the conformational change shouldn't happen before the HAs can aggregate and establish a state of restricted lipid flow. The free energy released by the formation of the extended coiled coil should be less than that required to form the hydrophobic defect by enough to give the free energy of activation needed to slow the step down adequately.

The free energy released by the formation of the extended coiled coil the size of that formed by HA is ~ -30 kcal/mol(HA) (Dieckmann et al., 1998; Kozlov and Chernomordik, 1998). Thus, the transformation of two or three HAs to extended coiled coils could provide up to -60 or -90 kcal/mol for the creation of the hydrophobic defect. Obviously, not all of this free energy may be available for defect stabilization, but that is not a practical concern at present.

The estimate for how much free energy is required to make fusion is more involved and less definite. To begin, little free energy should be required to move the fusion peptide from viral membrane to target membrane, since the hydrophobic portion of the peptide is embedded in a bilayer at both ends. Thus, the free energy released by the conformational change is only needed to create the hydrophobic defect in the outer monolayer of the viral membrane.

The next step is to estimate the size of this defect. Three embedded loops of an α -helical fusion peptide should displace two to three phospholipids. The simple depiction of the central HAs in the fusion site in Fig. 2 *B* shows only one fusion peptide per HA within the area of lipid flow restriction and the other two fusion peptides outside. This condition is likely to be general, since the hydrophobic defect will be small, only a few HAs within an aggregate could border it. It could also be that an HA has only one or two of its fusion peptides embedded, rather than all three, but this is not a practical point. It will be assumed that only one fusion peptide must be extracted from the site of restricted lipid flow per HA.

Since two to three HAs must undergo the essential conformational change to initiate fusion (Bentz, 2000), thereby removing two to three fusion peptides from site of restricted lipid flow, the size of the defect would be of the order of four to nine phospholipids, as depicted in Fig. 2 *B*. For simplicity, we can assume that the lipids are completely laterally displaced by the peptide, i.e., no tilting of acyl chains, which probably overestimates the surface area of the defect. We will consider the effect of tilted fusion peptides in the Discussion. Thus, a cylindrical defect of ~ 10 – 20 nm² would be formed, 1.5 nm sides (the length of the acyl chains) and a 1–1.4 nm radius, assuming smooth (not molecular) surfaces.

The final step is to estimate the free energy required to create a defect of this size. This estimate is even more crude, not only because the parameters for this type of "structure" are not known as rigorously, but also because the molecular surface of the hydrophobic defect must be a mix of acyl chains and hydrophobic amino acid side chains of the HA transmembrane domains. Ironically, it appears that this mixed lipid/protein surface may be required both by the closeness of packing of HAs required to restrict lipid flow and by the energetics of forming the hydrophobic defect. This provides a natural role for the transmembrane of fusion proteins.

We can begin with an underestimate of this free energy. The energy required to create a pore this size in a pure lipid monolayer, i.e., half that required for a bilayer, by osmotic shock or electric field pulse is ~ 40 – 55 kcal/mol, assuming standard values for surface tension, $\sigma = 2 \times 10^{-3}$ N/m, and edge energy, $\tilde{A} = 10^{-11}$ N (Winterhalter, 1999). It is clearly less than the -60 to -90 kcal/mol available from the HA conformational change, and this gets us over the first hurdle.

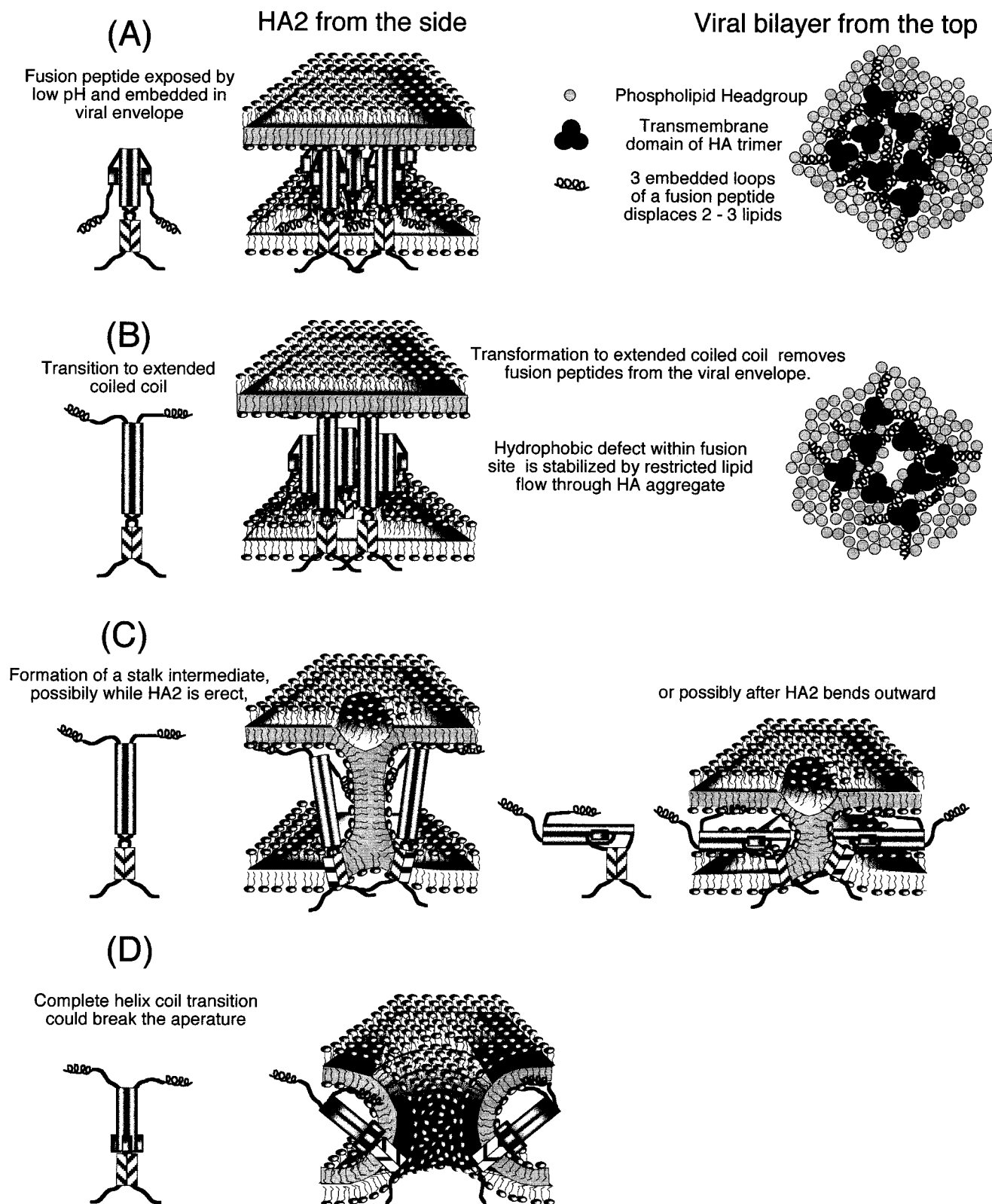


FIGURE 2 The hypothesis for HA-mediated fusion. Lipids and proteins are depicted to scale. Only the conformations of HA2 are shown, i.e., for visual clarity the HA1 headgroups are omitted. (A, left) The proposed conformation of HA2 after the fusion peptide is exposed, like Fig. (1, conformation 2). (A, middle) Side view of an aggregate of HAs, with their fusion peptides embedded into the viral envelope. (A, right) A view from the top of the HA-containing bilayer showing the lipid headgroups, the tops of the transmembrane domains, and the embedded fusion peptides. (B, left) The proposed conformation of

Now, we can obtain an overestimate for the free energy required. It is likely that the alkane/water interfacial tension value of $5 \times 10^{-2} \text{ J/m}^2 = 7.2 \text{ (kcal/mol-nm}^2\text{)}$ (Small, 1986) overestimates the water/acyl chain interaction within this defect, but it is a rigorous value. If the whole defect were purely lipidic, then the estimate for the free energy would be 84–142 kcal/mol, i.e., 24–52 kcal/mol more than the HA conformational change could provide. However, the hydrophobic defect is also composed of exposed transmembrane amino acid side chains. We can use the value of 2.3 kcal/mol-nm² of exposed nonpolar amino acid side chains to estimate their contribution (Wimley et al., 1996). Assuming a 1:1 composition of acyl chains and amino acid side chains on the sides of the cylindrical defect surface and pure lipid on its base (from the tail ends of the acyl chains of the inner monolayer), which is arbitrary but about right from Fig. 2 *B*, yields a free energy for formation of the hydrophobic defect of 62–110 kcal/mol. This overestimate is just a little more than the estimated available free energy from the conformational change to the extended coiled coil.

While these estimates are necessarily crude, they imply that the extraction of the virally embedded fusion peptides is energetically possible. It can be argued that not all of the energy from the protein conformational change might be available. However, it is also true that the transition time of the essential conformational change is at least five to seven orders of magnitude slower than expected for formation of a coiled coil, which implies an activation free energy of ~7–10 (or more) kcal/mol (Bentz, 2000). Thus, the bottom line is that the hydrophobic defect depicted in Fig. 2 *B* can be formed by two to three HAs undergoing the conformational change to the extended coiled coil, in part because the transmembrane domains of the HAs are part of this hydrophobic defect. This will be discussed further below.

To test whether the formation of the extended coiled coil creates a hydrophobic defect in the viral (or HA expressing cell bilayer) will be difficult, but a first step could entail the usage of detergents that do not impart significant curvature to the bilayers, such as bile salts, to determine whether they can block destabilization differently than lysoPC, which alters the intrinsic curvature of the monolayers (Chernomordik et al., 1997–1999). That is, these detergents would be expected to “heal” the hydrophobic defect, without hindering the ability of curvature-dependent lipid intermediates, e.g., stalks, proposed by others and below to mediate other steps of fusion. Obviously, a kinetic analysis would be

required to determine which fusion intermediate was affected.

Returning now to the hypothesis, the HAs surrounding the hydrophobic defect cannot immediately diffuse apart (thereby admitting lipids from the viral outer monolayer), since that would require reversing the aggregation mechanism and initially require expanding the surface area of hydrophobic defect by inclusion of pure acyl chains. The aggregate of HAs has become a dam holding the lipids on the outside. Clearly, this is a very high-energy intermediate that is stabilized by the restricted flow of lipid due to the HA aggregate (Tse et al., 1993; Zimmerberg et al., 1994; Chernomordik et al., 1998). The other HA-HA interactions that stabilize this “dam” are the exposed fusion peptides of HAs that haven’t yet undergone the conformational change to the extended coiled coil.

The next obvious question is what would be the half-time for maintenance of the restricted lipid flow state relative to the half-time for the excursion of lipid molecules from the target membrane. The only reasonable means of estimating this time is a very lengthy and expensive molecular dynamics simulation, given the number of atoms in (roughly) eight HAs, 200 phospholipids, and 10^4 or more water molecules. Even then, the half-times would be extremely sensitive to the distance of separation between the bilayers. While it would be desirable to know the energy cost of expanding this HA barrier enough to admit lipid flow, the expansion would occur on a molecular-size scale. A calculation based upon macroscopic theories would be quite unreliable and it wouldn’t yield a half-time of stability for the defect in any event. However, one element of this calculation seems predictable. It is likely that the free energy per unit area of exposure of acyl chains to water will remain greater than that for the side chains of transmembrane amino acids. Thus, expansion of the defect will be more energetically costly, per unit area, than its original formation, since expansion will come nearly completely from exposure of new acyl chains. Obviously a conclusive answer will require an extensive, well-planned hierarchical calculation, followed by the right experiments. It is also possible that the morphology of the hydrophobic defect could change, as the lipids facing it rearrange over time, without substantially reducing the free energy.

The hydrophobic defect can be relieved by recruitment of lipids from the outer monolayer of the apposed target membrane, aka the first step of fusion. This process would

HA2 once the transition to the extended coiled coil occurs for the HA monomers whose fusion peptides are within the fusion site, like Fig. (1, conformation 3). (*B, middle*) The space evacuated by the fusion peptides fills immediately outside the fusion site, but the acyl chains remain exposed within the fusion site due to the restricted lipid flow, i.e., depicting the hydrophobic defect. (*B, right*) The view from the top with this open space depicting the hydrophobic defect. (*C*) The formation of a lipidic stalk between the apposed membranes, formed by the lipids of the target membrane filling in the hydrophobic void. This stalk could form within HAs either standing up (on the left) or lying down (on the right, with a much lower free energy), perhaps facilitated by a helix-turn transition of one HA2 monomer. No view from the top is shown, as it is identical to the right side of (*B*). (*D*) The formation of the fusion site, with communication of aqueous compartments, perhaps due to the completion of the helix-turn conformational change.

probably start with movement of individual or small aggregates of lipids. How this happens isn't known; however, lipids show substantial positional fluctuations on a subnanosecond time scale (Husslein et al., 1998). It is possible that the fusion peptides now embedded in the target membrane would facilitate the movement of the lipids from the target membrane to the hydrophobic defect in the viral envelope (Durell et al., 1997). This molecular translocation would continue until a relatively stable contiguous structure forms between the outer monolayers.

For pure lipid systems the equilibrium formation of a lipid stalk appears to require the least energy for connecting apposed outer monolayers (Kozlov et al., 1989; Siegel, 1993a, b; Chernomordik et al., 1995, 1997). Chernomordik et al. (1998, 1999) and Kozlov and Chernomordik (1998) have proposed that the lipid stalk is an intermediate of HA-mediated fusion for this reason. However, within the HA fusion site, whether it forms from a nucleation of lipids, as proposed here, or by the continuous bending of lipid monolayers, as proposed by Kozlov and Chernomordik (1998), is a very important question, since its answer will show where the energy of the HA conformational change is first spent.

Fig. 2 *C* shows this stalk, either with the HAs erect (on the left, which seems too great a distance) or tilted (on the right). Tilting has been reported in reconstituted systems (Tatulian et al., 1995; Wharton et al., 1995; Gray and Tamm, 1997, 1998) and the energy cost of forming the shorter stalk would be much less. It could be that the helix-turn transition could cause this tilt, by occurring first for just one of the HA coils, as depicted on the right side of Fig. 2 *C*. That the crystal structure of TBHA2 shows all three helix-turns may simply reflect the symmetry of the fragment rather than a requirement for fusion. Again, it is not known that this helix-turn transition occurs on intact HA, but if it does, this model requires that HA remains upright through the formation of the extended coiled coil so that it can extract the embedded fusion peptide. This is structurally easy to believe from the arguments of Kozlov and Chernomordik (1998).

The final step in this process is the transformation of the stalk to form the fusion site, showing lipid mixing and then the contents mixing. Other intermediate steps are possible. While this step has been considered in some detail with pure lipid systems (Kozlov et al., 1989; Siegel, 1993a; Chernomordik et al., 1995), the fact that the hemifused state can be stabilized in HA-mediated fusion suggests that the protein strongly affects the process. The tension provided by this nascent helix-turn transition could facilitate the transformation of the stalk to a fusion pore. The helix-turn transition at the base of HA2, residues 104–112, if effected on the intact virus, would force the base of the HA trimer ectodomain down onto the top of its transmembrane domain (Fig. 1, conformation 4). Since the N-terminus of HA2 would now be tethered to the target membrane, this tension on the

transmembrane domain would tend to pull the stalk apart and open the fusion site, as depicted in Fig. 2 *D*.

DISCUSSION

This hypothesis provides a simple and direct transduction of the energy released by the formation of the coiled coil to the energy needed to create and stabilize the high-energy intermediates of fusion. The committed step in influenza HA-mediated fusion is proposed to begin with an aggregate of HAs (at least 8) with some of their HA2 N-termini, aka fusion peptides, embedded into the viral bilayer. These HAs are packed so closely that lipids from the viral envelope are restricted from diffusing into the center of the aggregate. The conformational change of HA to the extended coiled coil extracts the fusion peptides from the viral bilayer. When this extraction occurs from the center of the site of restricted lipid flow, it exposes acyl chains and parts of the HA transmembrane domains, i.e., the hydrophobic defect. This is the "transition state" of the committed step of fusion. It is stabilized by a "dam" of HAs, which are inhibited from diffusing away. Recruitment of lipids from the apposed target membrane can heal this hydrophobic defect, initiating lipid mixing and fusion.

The obvious question is whether the free energy needed to create the hydrophobic defect proposed in Fig. 2 *B* can be provided by the conformational change to the extended coiled coil. No rigorous answer is possible, but the crude estimate is yes, just about. Actually, the free energy supplied by conformational change should be several kcal/mol less than that required to form the hydrophobic defect, to make sure that the conformational change doesn't occur before an adequately large HA aggregate can form to sustain lipid flow restriction. While evolution could certainly have produced a more energetic conformational change for HA, e.g., by making the extended coiled coil longer, the fusion efficiency of that "faster" HA would be predicted by this model to diminish. This is a clear source of natural selection adequate to explain the high homology of the HA loop regions. In models where the formation of the extended coiled coil is assigned solely to bring the fusion peptide to the target membrane, the source of the natural selection needed to maintain the homology can only be theoretical, e.g., possible packing constraints or extra energy required for synthesis and folding. Of course, if the conformational change took much longer, the virion would never escape the endosomal/lysosomal pathway.

The HA transmembrane domains are required to be part of the hydrophobic defect because the HA aggregate must be closely packed enough to restrict lipid flow. Having the transmembrane domains as part of the hydrophobic defect also lowered the free energy required for its creation. This observation alone could explain the reported importance of transmembrane domain flexibility on viral fusion protein activity (Cleverley and Lenard, 1998). A rigid transmem-

brane domain should not participate in the defect surface as well, i.e., to lower its free energy of formation.

There is some morphological information about the HA fusion site. Kanaseki et al. (1997) have presented a wide array of beautiful electron micrographs of influenza virions (A/PR/8) fusing with glycophorin-containing liposomes. Unfortunately, there are no data showing the kinetics of fusion under the same conditions as the electron microscopy. Nevertheless, the fusion sites appear as protrusions from the outer monolayer of the liposome target membrane and fusion appears to start in the center of these sites with the liposome target membrane being pulled toward the viral membrane (see their Figs. 6 and 8). This would be expected from the model proposed here. These protrusions are of the order of 10 nm in diameter, which would be the diameter of a collar of roughly eight HAs, although the thickness of the shadowing makes quantitation very uncertain.

The “essential” conformational change for fusion

Of course, it remains to be proven that the formation of the extended coiled coil is the “essential” conformational change for the formation of the first fusion pore identified kinetically in Bentz (2000). As yet, no structural technique has succeeded in showing a rigorous correlation between the kinetics of fusion intermediate formation and the kinetics of extended coiled coil or helix-turn formation (Körte et al., 1997, 1999; Shangguan et al., 1998). Qiao et al. (1998) found that mutations predicted to hinder the extended coiled coil formation did inhibit fusion, suggesting that this conformational change might be necessary for fusion, although other conformational changes could have been responsible. Puri et al. (1990) showed that incubation of isolated virions (A/Japan/305/57 strain) at low pH resulted in no substantial morphological change in HA over the time range where virion fusion was studied. Shangguan et al. (1998) showed with the A/PR/8/34 strain that virions could fuse only before a morphological change consistent with the formation of the extended coiled coil was evident on the isolated virions. Körte et al. (1999) corroborated this finding with a cryo-electron micrograph of X31 virions, suggesting that loss of fusion activity correlated with the formation of extended coiled coil. Thus, the formation of extended coiled coil appeared not to be a *precursor* of fusion (Shangguan et al., 1998). If the extended coiled coil is necessary for fusion, then destabilization must be coupled directly to the motion of the conformational change.

The time constant for the “essential” conformational change leading to the first fusion pore was found to be $\sim 10^4$ s, on average (Bentz, 2000). The morphological changes indicative of formation of the extended coiled coil on the isolated PR8 virions required $\sim 10^3$ s (Shangguan et al., 1998). However, the cell lines used in Melikyan et al. (1995a) express the Japan strain of HA, which has slower and less complete inactivation kinetics than PR8 (Puri et al.,

1990; Körte et al., 1997, 1999). Thus, the similarity of time scales is striking. This suggests that the extended coiled coil morphology visualized in Shangguan et al. (1998) is due to the essential conformational change for first-fusion pore formation.

How can fusion happen in 100–200 s, when the “essential” conformational change for HA occurs only in 10^4 s, on average. The reason is that HA refolding kinetics must be broadly distributed, i.e., some sooner and some later, and that fusion experiments of Melikyan et al. (1995a) only monitor the very *first* of hundreds or thousands of fusogenic HA aggregates (depending upon HA surface density), which succeed in forming the first fusion pore (Bentz, 2000). The essence of any fusion experiment is to observe only the first of a large ensemble of HA aggregates to succeed.

Integration of fusion models

Kozlov and Chernomordik (1998) have proposed a mechanism for HA aggregation and fusion that starts with the fusion peptide embedded in the viral envelope. They estimate that while one HA could not deform the membrane, an aggregate could after one or more of the heptad repeats of the extended coiled coil form, thereby holding the membrane up in a locally curved state which is held in place by the binding strength of the heptad repeats. This membrane deformation should continue, as more heptad repeats form in the extended coiled coil, until the curvature energy exceeds the binding energy of the next heptad repeat. The HAs should become tightly packed, i.e. perhaps until the state of restricted lipid flow is reached.

Their model continues by proposing that the six or more HAs within the aggregate (with fusion peptides embedded in the viral envelope) simultaneously tilt away from the center, forming a dimple of bilayer that is reaching toward the target membrane with a great deal of bending energy at the tip. They argue that this tilting does not cost much energy and propose that this structure continues to grow from the viral bilayer until it forms a “stalk” connecting the outer bilayers.

However, the model of Kozlov and Chernomordik (1998) has no role for the state of restricted lipid flow and no obvious explanation for the fact that only two or three HAs within the aggregate need to transform slowly in order for the first fusion pore to form (Bentz, 2000). It could be argued that the remaining four or three, or more, HAs tilt rapidly and cooperatively, but that doesn’t appear compatible with their calculation of the mechanical forces needed to create and stabilize the dimple. Furthermore, for the dimple to grow toward the target membrane requires more lipids to flow into the site from the viral bilayer, which wouldn’t occur if the lipid flow becomes restricted.

There is an appealing combination of these two models. Beginning with the model of Kozlov and Chernomordik

(1998), the HAs in the fusion site will tilt and begin to form the dimple toward the target membrane. The collar of HAs will tighten until the site becomes lipid-flow-restricted. Then the dimple can grow no further. The tension on the fusion peptides will cause some of them to pull out the viral envelope (perhaps one hydrophobic residue at a time, which should require less energy or force than removal of the whole peptide at once), and move to the target membrane following the formation of the extended coiled coil. This produces the unstable site shown in Fig. 2 *B*. The stalk proposed by Kozlov and Chernomordik (1998) could assemble from the lipids moving from the target membrane to heal the hydrophobic defect.

An experimental dissection between the model proposed by Kozlov and Chernomordik (1998) and the one proposed here would begin with increasing the hydrophobicity of the fusion peptide, e.g., mutants with more hydrophobic side chains on the appropriate amino acids of the fusion peptide. The model of Kozlov and Chernomordik (1998) would predict equal or better fusion. The model proposed here would predict that eventually fusion would diminish, as the fusion peptides became too hydrophobic to be pulled out by the extended coiled coil. Interestingly, Qiao et al. (1999) found that point mutations of the HA2 N-terminal glycine, at the end of the fusion peptide, to more hydrophobic residues reduced (for G1A) and abolished (for G1V) fusion of HA expressing cells. More hydrophilic residues also reduced (G1S) or abolished (G1E) fusion, although perhaps for different reasons. Further studies will be needed before any conclusions can be reached. In this regard, the work suggesting that viral fusion peptides appear to embed in bilayers at an oblique angle (Peuvot et al., 1999), if correct for the intact HA during fusion, may well reflect an optimization between maximizing the "footprint" of the peptide in the viral outer monolayer, to create the largest defect upon extraction, while maintaining the hydrophobicity of the fusion peptide insertion, to allow extraction with the required kinetics to sustain fusion.

Resolution of paradoxical results

The fusion model proposed here is consistent with both the common results of HA-mediated fusion and with the paradoxical results. A recent interesting result is that HA expressing cells lose fusogenicity when they are inflated at 24°C, but not at 32°C (Markosyan et al., 1999). Their explanation is that bilayer expansion and thinning causes an inhibition of the dimpling of the membrane before stalk formation, along the lines of the model of Kozlov and Chernomordik (1998). However, the hypothesis presented here gives an alternative and testable explanation. Penetration of peptides into lipid monolayers shows increased binding as surface pressure is reduced (Rafalski et al., 1991). This suggests that embedded peptides will require increased energy to be extracted from the expanded bilayer-

ers, which would certainly inhibit fusion by this hypothesis. The energy balance estimates presented above strongly suggest that a modest increase in the binding constant of the peptide could defeat the efforts of the extended coiled coil to extract the embedded fusion peptide. Furthermore, it seems likely that peptide binding would decrease with increasing temperature, which would be consistent with the data of Markosyan et al. (1999), if peptide extraction is the explanation.

A truly paradoxical result was found in Alford et al. (1994). The kinetics of fusion of influenza virus with phosphatidylethanolamine (PE)/phosphatidylcholine (PC)/glycophorin liposomes did not respond to the temperature of hexagonal H_{II} phase transition temperature, T_H , of the lipids composing the target membrane. If fusion required bilayer-bilayer contact, then the fusion rate should have increased at T_H (Bentz et al., 1987). Thus, the fusion between the virions and the glycophorin containing liposomes did not appear to require bilayer-bilayer contact, a suggestion at odds with other models of HA-mediated fusion. Furthermore, the fusion kinetics were the same whether the target membrane lipids were pure PE or (1:2) PE/PC, and were much slower when the target membrane lipids were pure PC. Because the fusion kinetics did not depend smoothly on PC/PE composition, the fusion mechanism appeared indifferent to the known material properties of the lipid bilayers (Ellens et al., 1989; Alford et al., 1994), a suggestion even more at odds with other models of HA-mediated fusion. The hypothesis proposed here eliminates these inconsistencies, since the initial contact would occur between a hydrophobic defect and the target membrane, which would not depend upon the energetics of close approach of the lipid headgroups.

This hypothesis for protein-mediated fusion by coiled coils largely depends upon exposed hydrophobic surface, like that proposed for the fusion of pure PC liposomes (Lee and Lentz, 1997). The highly curved bilayers of sonicated PC liposomes (30 nm diameter) have substantial surface exposure of acyl chains and they will fuse spontaneously after polyethyleneglycol (PEG)-induced aggregation. Large unilamellar liposomes (LUV, 100 nm diameter) made of pure PC do not show fusion. However, Lentz et al. (1998) showed that LUVs with some of the outer monolayer lipids removed showed limited fusion. Furthermore, while the fusion peptide of HA is required for fusion, the free peptide alone inhibits the fusion of PC LUV, probably by decreasing the bilayer surface hydrophobicity of the PC membranes (Lentz et al., 1998). Likewise, the free peptide alone inhibits the fusion of predominantly PE membranes, probably by increasing the spontaneous radius of monolayer curvature of these bilayers (Siegel and Epand, 1998). In both cases, the free peptide will embed in the liposome bilayer and stay there. The hypothesis proposed here centers on fusion being due to removing the fusion peptide from the viral envelope *at the right time*.

It was believed that the HA fusion reaction should be nonleaky, until recent experiments proved otherwise (Shangguan et al., 1996; Blumenthal and Morris, 1999). There remains a belief that this leakage is an experimental artifact, not relevant to the virion. However, the fusion mechanism proposed here could generate leakage easily. The important question is whether the same site can leak and then reseal by fusion, or whether some sites fuse while other sites lyse.

The extent to which HA-mediated fusion is leaky appears to depend upon the assay and the membrane system. For example, Shangguan et al. (1996) found that influenza virions fusing with 0.1 μm PC/ganglioside liposomes was lytic, i.e., all contents (up to 10 kD dextran) were lost at the same time as lipid mixing. Leakage of calcein from liposomes induced by influenza virus was evident in the data of Günther-Ausborn et al. (1995), although this result wasn't discussed. Chernomordik et al. (1997, 1998) found that HA expressing cells fusing with dye-loaded erythrocyte ghosts showed substantial, but not necessarily complete, contents mixing. Leakage was not specifically monitored. Blumenthal and Morris (1999) detected leakage with basically the same system, either at the same time as lipid mixing (for some cells) or delayed (for other cells).

Blumenthal and Morris (1999) speculate that fusion of the first site is nonleaky and leakage is due to "damage" from subsequent fusion sites forming. However, on average, leakage by any mechanism should be delayed compared with lipid mixing as the size of the area of contact between the apposing membranes increases, since leakage requires escape from the area of contact while lipid mixing is local. If the fusion site first leaks and then reseals following fusion, then the same observations would be made. If the fusion site is near the edge of the area of apposition, then leakage would occur at the same time as lipid mixing. If the fusion site is near the center of the area of apposition, then leakage would occur later than lipid mixing. The area of apposition between a virion and a liposome is obviously much smaller than that between an HA expressing cell and an erythrocyte, hence leakage from an LUV would be expected to be coincident with lipid mixing with the virion if the fusion site leaks then fuses. Furthermore, a 0.1 μm liposome could lose its entire contents ($\sim 0.5 \times 10^{-18}$ l) during the process, while the same volume loss from an erythrocyte would be insignificant. This can explain the observation of Shangguan et al. (1996). Qiao et al. (1999) observed leakage after first fusion pore formation, via whole cell capacitance measurements, but the reverse sequence could probably not be observed since leakage would overwhelm the first fusion pore signal. Thus, the kinetic analysis of the leakage data is not yet refined enough to determine whether or not leakage and fusion happen at different sites.

Some data cannot be explained by the hypothesis. Liposome fusion has been induced by an 11-amino acid synthetic peptide when tethered to a lipid (Martin et al., 1999),

and Epand et al. (1999) have claimed that a fragment of HA with a preformed extended coiled coil can induce liposomes with a nearly isotropic phase composition of lipids to undergo limited liposome-liposome lipid mixing. It remains to be shown that these systems follow the same path for bilayer destabilization as full-length HA in a membrane.

Extension of the hypothesis to other fusion proteins

Since other viral fusion proteins show evidence of extended coiled coils as part of their core equilibrium structures, it has been widely speculated that a basic motif of viral fusion proteins involves this "spring-loaded" conformational change (Carr and Kim, 1993; Bullough et al., 1994; Carr et al., 1997; Caffrey et al., 1998; Weissenhorn et al., 1997, 1998; Skehel and Wiley, 1998; Baker et al., 1999; Singh et al., 1999). Two key revisions to that speculation are proposed here: 1) that the initial defect (which requires high free energy) is formed by the conformational change to the extended coiled coil (Figs. 1, conformation 3; and 2 B), which should release the most free energy; and 2) the helix-turn transition to form the annular helical domains (assuming it occurs) should be responsible for a lower-energy process, such as bending the protein to facilitate the formation of the lipidic stalk or/and rupturing the stalk structure to permit lipid and aqueous contents mixing.

The robustness of this hypothesis can be seen by considering the paramyxovirus simian parainfluenza virus 5 fusion protein (Baker et al., 1999), denoted SV5 F, whose core ectoplasmic fragment was recently solved. This is a good example because it is rather different from HA. The authors note that this SV5 F fragment, while showing a very similar coiled coil motif as that found with HA2, gp41, and other viral fusion protein cores, does differ in three respects, which they suggest implies a novel model for driving the fusion reaction. These differences in the structure of the SV5 F protein are (Baker et al., 1999):

1. The core coiled coil structure appears to extend into the fusion peptide of the F protein, rather than there being a linker between the N-terminal heptad repeat domain and the fusion peptide. Chen et al. (1999) have observed a lengthening of the extended coiled coil for an HA fragment using a different expression system, although it doesn't yet reach the fusion peptide;
2. There is a small linker between the transmembrane domain and the C-terminus of the core coiled coil domain, which can be deleted, as opposed to longer linkers for the other fusion proteins. Interestingly, McNew et al. (1999) have extended the linker in the SNARE fusion system, discussed further below, and found that fusion diminished;
3. There is a long intervening polypeptide chain (~ 250 AA) between the two heptad repeat domains, as opposed to no or small ordered intervening chains.

While it is not known how these structural differences may affect fusion, they appear much less significant in light of the model proposed here assuming, of course, that SV5 F does fuse like HA. In the first case, if the extended coiled coil forms after the fusion peptide has been removed from the viral envelope, then the overlap of the coiled coil domain and the fusion peptide would only affect the evolution of the nascent fusion site, not its initiation. Thus, the primary function of the extended coiled coil remains the same. In the second case, the linker to the transmembrane domain is important to the model proposed here only when it becomes so long that the apposed membranes are too far apart for the lipids from the target membrane to reach the hydrophobic defect in time. The helix turn could cleave the lipidic stalk, as speculated in Fig. 2 *D*, and a shorter linker might facilitate this process. In the third case, the long intervening polypeptide chain may simply facilitate the tilting of the protein, as suggested by Baker et al. (1999) and shown schematically in Fig. 2 *C*, on the right.

A model for fusion mediated by coiled coil proteins must be able to explain the ability of peptides homologous to coiled coils, aka heptad repeat domains, to inhibit fusion (Wild et al., 1994; Jiang et al., 1993; Lu et al., 1994; Munoz-Barroso et al., 1998; Joshi et al., 1998). These peptides are thought to competitively inhibit the formation of the extended coiled coils and/or the helix-turn domains.

We can focus on the peptide inhibition data for the SV5 viral fusion system since no peptide inhibition data has been published for HA and the data for SV5 are more detailed than other studies. Joshi et al. (1998) found that peptides of the N-terminal heptad repeat region could block aqueous contents mixing between CV-1 cells expressing the SV5 F protein and erythrocytes, but not lipid mixing, whereas peptides of the C-terminal heptad repeat region could block aqueous contents and lipid mixing between the cells. Their explanation was that the C-terminal peptides bind to the N-terminal heptad repeat domain of the protein in order to block the first step of fusion, i.e., lipid mixing, and the N-terminal peptides bind to the C-terminal heptad repeat domain of the protein, in order to block the second step of fusion, i.e., contents mixing. This explanation is contrary to the common assumption that the C-terminus peptides bind to the C-terminus heptad repeat binding site and the N-terminal peptides bind to the N-terminus heptad repeat domain.

The model proposed here yields a more natural explanation assuming, of course, that native SV5 F has the same general orientation and behavior as HA. The C-terminal peptides should block the “helix-turn” element of the fusion mechanism proposed here. How does this block lipid and content mixing, since it wouldn’t block the formation of the extended coiled coil? Recall that the formation of the extended coiled coil is only the first (and high-energy) transition and it only creates the first defect. If the subsequent steps, like the helix turn, are inhibited, then the nascent

fusion site will simply dissipate, as lipids eventually flow into it. This would certainly block contents mixing and the lipid mixing between the apposed membranes.

However, the N-terminal peptides should block the “extended coiled coil” formation of this fusion mechanism which, at first glance, would be expected to block all interactions between the membranes, even with the model proposed by Joshi et al. (1998) and Baker et al. (1999). However, the formation of the extended coiled coil may not be easy to stop because it involves so much free energy. Qiao et al. (1998) found that to abolish fusion of HA expressing cells required two prolines to be expressed appropriately within the coiled coil sequence to inhibit fusion. Furthermore, the N-terminal portion of the protein should not be accessible for very long for binding by the N-terminal peptides, since the extended coiled coil formation is cooperative and it occurs within a protein aggregate. Once formed, the extended coiled coil has done much of its job, even if excess peptide could pry it apart. These competing effects would reduce binding of the N-terminal peptide and, therefore, the number of proteins capable of forming extended coiled coils would be reduced, but not necessarily eliminated. Chernomordik et al. (1998) have shown that reducing the cell surface density of HAs stops contents mixing, while lipid mixing continues via hemifusion. This explanation is speculative, but it is a natural consequence of the fusion model proposed here and it maintains the expected topology of N- and C-terminal peptide binding to the appropriate heptad repeat domains of the fusion protein.

With HIV, Munoz-Barroso et al. (1998) found that the C-terminal peptide of the fusion protein Env would block contents mixing, but not lipid mixing. While reverse to the SV5 F system, this result may suggest that the “helix-turn” transitions can be partially blocked adequately to stop contents mixing, but not some lipid mixing. Eckert et al. (1999) constructed D-peptides complementary to the highly conserved helix-turn binding pocket of env’s C-terminus which blocked infection and syncytia formation, in accord with the mechanism proposed here.

However, Env may look and act differently from HA. For example, data suggest that the HA molecules involved in cell surface attachment via HA1-sialic acid binding are not involved in fusion or are very slow (Ellens et al., 1990; Bentz, 1992; Alford et al., 1994). Millar et al. (1999) found that HAs bound to Fab linked to target membrane can eventually mediate fusion. The primary route of HIV infection usually starts with binding of Env to CD4, but it is the subsequent binding of Env to a chemokine receptor protein that is required for fusion (Feng et al., 1996; Doms and Peiper, 1998; Xiao et al., 1999). In fact, there is an Env mutant that binds directly to either chemokine receptor, CXCR4 and CRC5, and mediates fusion (Hoffman et al., 1999).

These chemokine receptors have seven transmembrane domains. While the type of chemokine receptor bound by

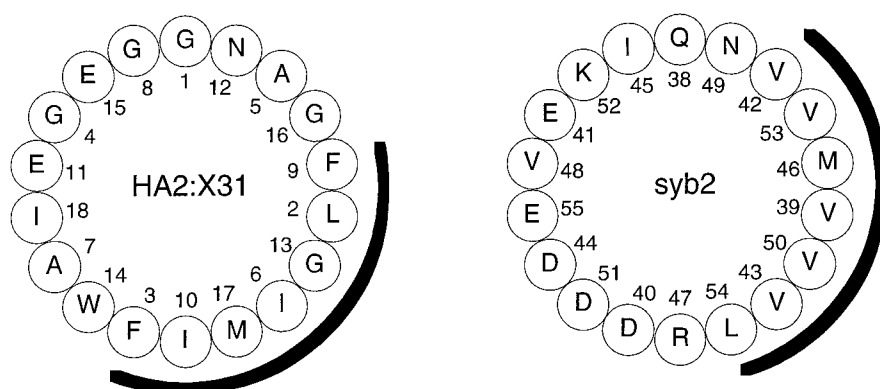


FIGURE 3 The possible “fusion peptide” of the v-snares. Helical wheel representation of the N-terminus of HA2, showing the hydrophobic face of the fusion peptide with the black band, and residues 38–55 of synaptobrevin 2, which is the v-snare cognate for the t-snares syntaxin and SNAP25 (Weber et al., 1998). Syb2 could have a “fusion peptide” by this criteria, which would allow this eukaryotic fusion system to function according to the same model as proposed for HA. These residues form the center stretch of the coiled coil core complex of syb2/syntaxin/SNAP25, just N-terminal of the central arginine, R56 (Sutton et al., 1998), which would form after vesicle docking to the target membrane. Other v-snares show similar hydrophobic faces on helical wheel representations, e.g., regions 35–52 of snc1, 172–189 of Nyv1, and to a lesser extent regions 137–154 of sar1 and 138–155 of sec22.

Env is important for the progression of HIV infection from macrophages to T-cells (Doms and Peiper, 1998; Xiao et al., 1999), it is clear how the recruitment of proteins with seven transmembrane domains into the collar of proteins at the fusion site could facilitate aggregation of Env, possibly by the mechanism proposed by Kozlov and Chernomordik (1998). Fusion could then proceed as depicted for HA. Even more intriguing is the possibility that these chemokine receptors themselves create a site of restricted lipid flow in the *target* membrane. If the “fusion peptide” of Env embedded initially in the target membrane at a site of lipid flow restriction, due to preliminary conformational changes, then extraction by the formation of an extended coiled coil would initiate destabilization from the target, rather than the viral bilayer. Analysis of this fusion system can determine the stoichiometry of the proteins at the fusion site (Bentz, 2000), which will be essential to sorting out how the fusion actually occurs.

Finally, we can consider eukaryotic fusion. While eukaryotic fusion may entail many elements (Mayer, 1999), the “minimal” v-SNARE/t-SNARE cognates proposed by Rothman and co-workers (Söllner et al., 1993; Weber et al., 1998; McNew et al., 1999) shows a coiled coil core structure similar to that of the viruses (Skehel and Wiley, 1998; Baker et al., 1999). However, no “fusion peptide” (amphipathic α -helix or otherwise) has yet been proven, which is an essential element of the model proposed here. Nevertheless, it is possible that the v-snares do contain a “fusion peptide.” Jahn and Südhof (1994) Fig. 3 shows a helical wheel representation of the fusion peptide of HA and of an 18-amino acid sequence of synaptobrevin 2, syb2, which is the v-snare protein associated with the t-snares syntaxin and SNAP 25 (Weber et al., 1998), whose core coiled coil complex has been recently solved (Sutton et al., 1998). The black band highlights the hydrophobic face of both peptides

and it is clear that syb2 shows a “fusion peptide” profile. This stretch (amino acids 38–55) occupy the center of the syb2 coiled coil domain in the crystallized 7S core complex, just N-terminal to the central arginine (R56) of the 7S complex (Sutton et al., 1998).

Before the docking of the v-snare syb-2 with the t-snare complex, syb2 is relatively unstructured (Fasshauer et al., 1997; Götte and Fischer von Mollard, 1998). Thus, it could be that this stretch of amino acids plays two roles in its life cycle, depicted in Fig. 4: 1) as a “fusion peptide” embedded in the secretory vesicle’s outer membrane until docking with the target membrane; and 2) as a piece of the new four-stranded coiled coil formed by syb2, syntaxin, and the N- and C-termini of SNAP25 after docking. The formation of the new coiled coil would pull syb2 out of the vesicle membrane and, if vesicular membrane proteins restrict lipid flow, then this eukaryotic fusion system could work the same way as HA. The other v-snare cognates (Weimbs et al., 1998) show similar “fusion peptide” profiles on the helical wheel for the aligned 18-amino acid sequences N-terminal to the central arginines, while the t-snare cognates do not (data not shown). McNew et al. (1999) found that adding peptide linkers between the transmembrane domain and the core complex coiled coil domain for either the v-snare VAMP-2 (aka syb-2) or the t-snare member syntaxin 1A decreased fusion in their reconstituted system. While there are several explanations for this result, the model proposed here would predict this result also. The linkers would allow the apposed membranes to be further apart after the formation of the new coiled coil, thereby making it more difficult for the target bilayer lipids to reach the hydrophobic defect. Finally, while neurosynaptic secretion is nonleaky and this fusion mechanism is to some extent leaky, there is no essential contradiction. The resolution could be that there are other proteins attached periph-

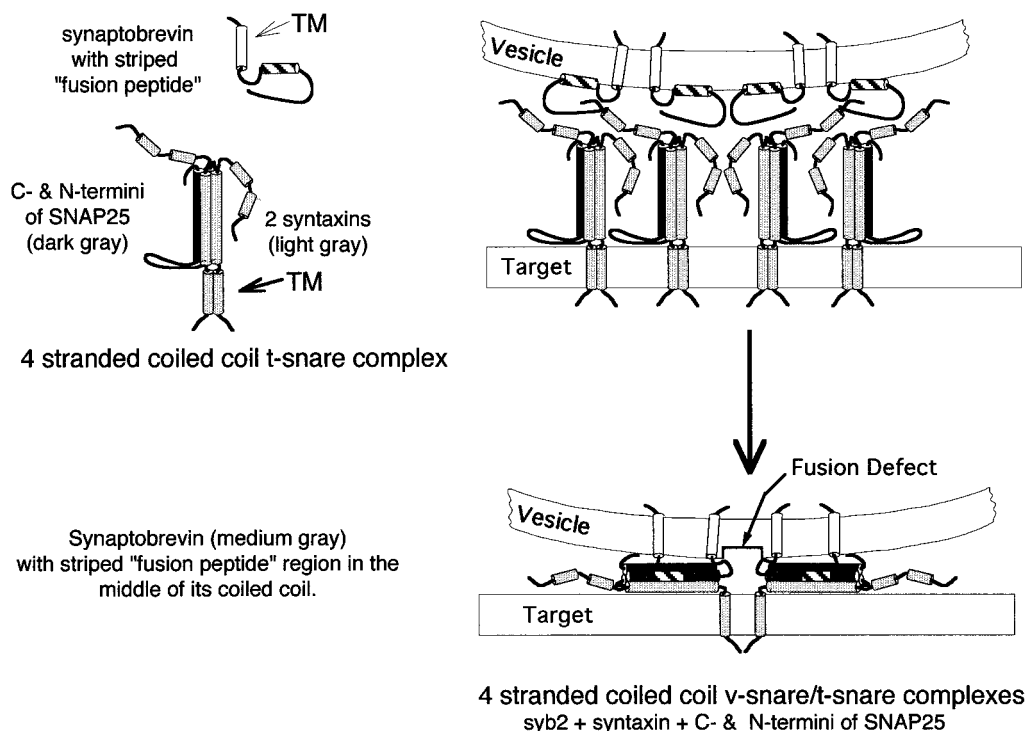


FIGURE 4 A proposed model for SNARE protein complex mediated fusion. The four coil bundle of two syntaxins and one SNAP25, with heptad repeats at both N- and C- termini, are shown on the target membrane before binding of syb2 from the vesicle membrane (adapted from Götte and Fischer von Mollard, 1998). syb2 is relatively unstructured before interaction with the t-snares. Thus, the "fusion peptide" of syb2 could be initially embedded in the vesicle bilayer, like HA2 after acidification. Upon binding, the heptad repeat sequence of syb2 replaces one of the syntaxins in the four-helix bundle. The formation of the coiled coil of syb2 would pull the "fusion peptide" out of the vesicle bilayer, just as proposed for HA.

erally to the fusion site whose job is to contain the leakage (Bentz et al., 1990).

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