# Relationships Between Protein Domains and Lipid Monolayers in Membrane Fusion

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*Abstract*

The hemagglutinin (HA) of influenza virus is the prototypic viral fusion protein. The possible roles of each domain of HA in fusion are presented. Specifically, it is proposed that the ectodomain causes hemifusion, the transmembrane domain causes fusion pore formation, and the cytoplasmic tail causes pore flickering. Lipids must also participate in fusion. Pores are created in stable hemifusion diaphragms by increasing the spontaneous curvature of inner monolayers of membranes to be more positive, but further increase in spontaneous curvature does not promote pore growth. In contrast, increasing spontaneous curvature of inner leaflets does promote pore enlargement for wild type HA, demonstrating that there is a lipid component to pore growth.

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## Introduction

Biological membrane fusion occurs when two distinct membranes merge into one, allowing mixing of their constituent lipids and integral membrane proteins, and formerly separated aqueous compartments become joined, allowing mixing of their contents through a fusion pore. It has long been hypothesized that between membrane binding and initiation of a fusion pore is a key intermediate step, termed hemifusion (Palade, 1975). Hemifusion is defined as the continuity of initially

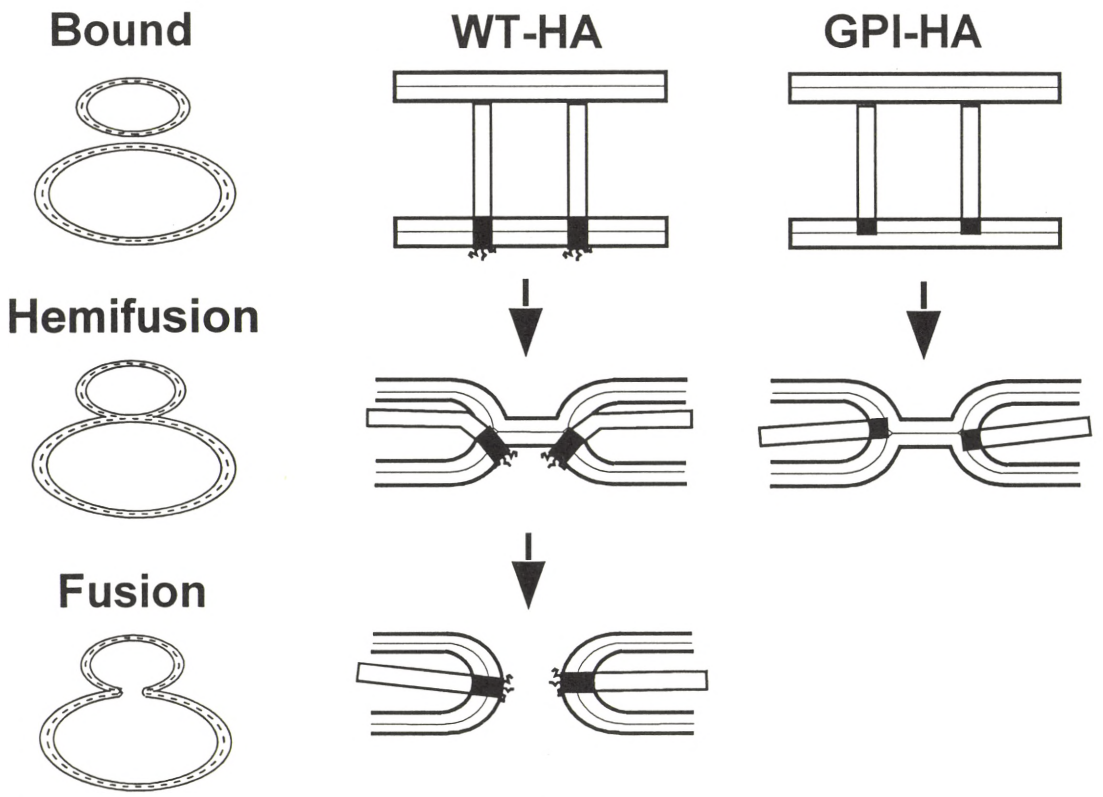


**Figure 1.** Schematic illustration of the membrane fusion process involving a stage of hemifusion.

contacting (for cellular fusion, outer) membrane monolayers, while noncontacting (inner) monolayers remain distinct but apposed and form what is called a hemifusion diaphragm (Fig. 1, left-hand panel). Hemagglutinin (HA) of influenza virus is the best characterized fusion protein (Wiley and Skehel, 1987; Bullough et al., 1994; Hernandez et al., 1996). In this paper, we review evidence from our laboratories to argue that separate domains of HA in particular, and possibly for fusion proteins in general, control sequential steps in fusion, including hemifusion.

HA is responsible for both binding and fusion. Influenza virus is internalized within cells by endocytosis and the low pH of the endosomes triggers fusion by causing conformational changes in HA (Gaudin et al., 1995). The fusion that occurs in this natural environment is modeled by expressing HA on cell surfaces, binding these cells to target membranes, and inducing fusion by lowering pH. HA is assembled from three identical monomers, each synthesized as a single polypeptide chain. Each monomer consists of about 550 amino acids (the precise number depends upon the strain) that can be divided into three domains: the ectodomain of some 515 amino acid residues, the transmembrane (TM) domain of some 27 residues, and the intraviral domain of 10-11 residues (Fig. 2). The intraviral domain is located in the cytoplasm when HA is expressed in cells, hence is referred to as the cytoplasmic tail (CT). The ectodomain is located in the extracellular space and HA-expressing cells interact extracellularly with their target membranes.

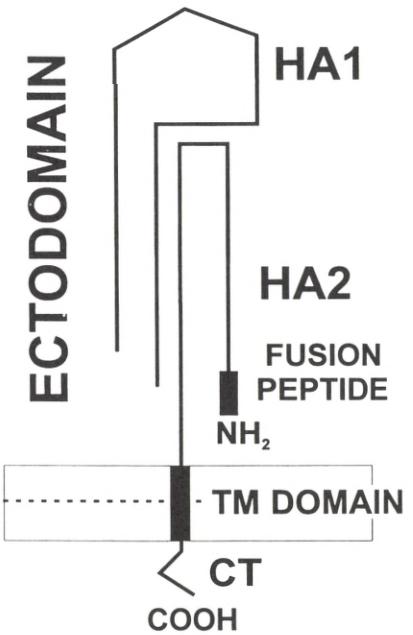


**Figure 2.** Hemagglutinin (HA) fusion protein of influenza virus.

## The Ectodomain of HA Induces Hemifusion

The ectodomain of HA has been molecularly engineered to anchor to membranes by a glycosyl linkage to a phospholipid (i.e. it is GPI-coupled) rather than attached to a TM domain (Kemble et al., 1993). Using this GPI-HA construct, we showed that hemifusion resulted without the subsequent formation of fusion pores (Kemble et al., 1994; Melikyan et al., 1995). We conceive that the process of hemifusion occurs as follows: The two distinct outer membrane leaflets come into contact and merge, allowing their lipids to mix. The high curvature of the initial connecting structure (known as a "stalk") causes these merging leaflets to move outward from the point of contact. With these leaflets cleared from the former contact point, the inner leaflets are now able to appose each other, forming a new lipid bilayer membrane, referred to as a "hemifusion diaphragm." The aqueous compartments are now separated by only a single bilayer. Any integral membrane proteins remain in the undisturbed portion of their original membranes because the portion of the TM domain that spans an inner leaflet is not able to enter the inner leaflet of its hemifused partner; inner leaflets remain unaltered and distinct (Fig. 1). The extracellular portion and CT of these proteins continue to reside in their respective aqueous spaces. At this stage hemifusion is complete. From the standpoint of lipid rearrangement, all that remains in order for fusion to be accomplished is the formation of a pore in the hemifusion diaphragm (HD). Since the GPI-HA construct, which isolates the behavior of the ectodomain, shows the ectodomain of HA is capable of inducing hemifusion, we can expect that the ectodomain of wild type also induces hemifusion.

## The CT of HA Controls Pore Flickering, but not Pore Formation

We know that the CT is not required for fusion: virus constructed to be void of the CT of HA (CT-minus) still produces fully infectious particles (Simpson and Lamb, 1992; Jin et al., 1994; 1996). Furthermore, cells expressing CT-minus fuse both to red blood cells (RBCs) and to planar membranes with the same kinetics as wild type HA (WT HA) (Melikyan et al., 1997b). Generally, when pores first form they flicker open and closed before fully opening, a common behavior of biological fusion pores . We have found that the CT controls flickering (Melikyan et al., 1997b): For CT-minus HA, pore flickering occurred twenty times less frequently than for WT. In WT HA, the CT is palmitoylated on conserved cysteines. Palmitoyls on the CT of HA are essential for significant amounts of pore flickering: mutating these cysteines to prevent palmitoylation also greatly reduced the degree of pore flickering. In other words, a palmitoylated CT promotes pore closing, but does not affect pore opening. Since the CT does not aid fusion and the ectodomain only yields hemifusion, the TM domain must be essential for full fusion.

## The Linkage Model of Fusion

What would account for the observation that the GPI-HA construct terminates in stable hemifusion while HA with an intact TM domain causes full fusion? It is known that upon exposure to low pH, the ectodomain of each monomer of HA dramatically reconfigures. In the case of WT HA, the massive conformational changes of the ectodomain in response to low pH should cause the small TM domain to which it is attached to move. Because HA should be surrounding the rim of the HD it creates, we conjecture that ectodomain movement pushes the TM domain into the HD as it is forming. Any forced insertion of the TM domain would disturb the HD: the TM domain would no longer be in its natural membrane-spanning orientation. The stresses created would be relieved if the lipids reconfigured around the TM domain into a new single bilayer, thereby reestablishing the TM domain's energetically favored orientation. For this to occur, a fusion pore would have to form (Fig. 1, WT-HA). Because the central conjecture of this model is that conformational changes of the ectodomain are linked to movements of the TM domain, we refer to it as the "linkage model" (Melikyan et al., 1995) In contrast to WT HA, for GPI-HA the lipid anchoring the ectodomain is free to move throughout the continuous outer leaflets upon hemifusion, thus the HD is not disrupted and fusion pores do not form (Fig. 1, GPI-HA, right-hand panels).

The linkage model has a common-sense logic in that function of each HA domain follows naturally from topology: in terms of cellular membranes, fusion proceeds outside-to-inside sequentially; each HA domain acts on the portion of the membrane to which it is in immediate proximity – the ectodomain merges outer leaflets, the TM domain spans and disrupts inner leaflets, and the CT, inside the cell, acts only after initial pore formation. Moreover, since we know the TM domain is linked to the ectodomain and that the ectodomain is moving extensively, it would seem imperative that the TM domain must also be moving to some degree; the TM domain is positioned just outside the HD, in the precise location one would expect for a structure that would be disrupting an HD.

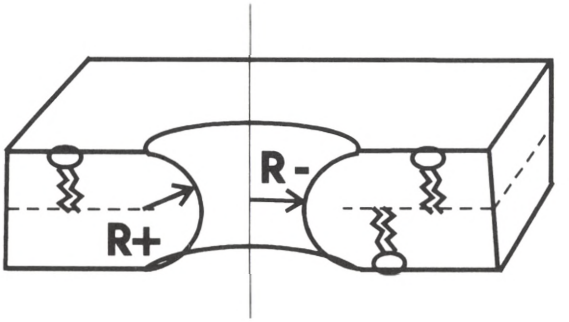


**Figure 3.** A lipidic pore has both negative ($R_-$) and positive ($R_+$) curvature.

## Increasing Positive Spontaneous Curvatures of Inner Leaflets Promotes Pore Formation within Hemifusion Diaphragms

In biological fusion, the role of the lipids as well as the proteins must be considered: at a minimum there is a change in their configuration as two bilayers merge into one. The manner by which lipids transiently leave their bilayer configuration is central to the fusion process. Any rearrangements that lipids undergo have to involve elastic energy which is required to bend the lipids out of the curvature of their original leaflets (Helfrich, 1973). A lipidic pore within an HD has a positive and negative curvature (Fig. 3). The positive curvature is expected to dominate, based on a straightforward application of surface geometry (Kozlov et al., 1989). Several laboratories have studied the consequences of altering spontaneous curvature of outer leaflets in protein-mediated fusion systems (Günter-Ausborn et al., 1995; Chernomordik et al., 1995; Shangguan et al., 1996), but because of their inaccessibility, inner leaflets – of which an HD is comprised – had not been studied in this manner. We surmounted this problem by adding, to solution, membrane permeable cationic agents (MPCAs) that are surface active. In this simple manner we were able to gain access to inner leaflets. These positively-charged micelle-forming agents preferentially insert into inner leaflets (Sheetz and Singer, 1974; Steck, 1989) (because inner leaflets are more negatively charged than outer ones) and promote formation of positive curvature structures (Hornby and Cullis, 1981).

GPI-HA-cells were hemifused to RBCs. The effect of adding MPCAs was the creation of pores that were highly targeted to the HD. We showed that fusion was caused by the positive spontaneous curvature agents acting directly upon inner leaflets (Melikyan et al., 1997a). Because the HD should be devoid of integral membrane proteins, the MPCAs could promote pore formation within lipid bilayers by a mechanism similar to that in an HD. In fact, we showed that the ability of MPCAs to bend into a positive curvature in planar bilayers directly paralled their ability to induce full fusion from a state of hemifusion. If in fact a fusion pore is a basically lipidic structure, then it is reasonable to conjecture that the TM domain of HA may induce pore formation through control of the spontaneous curvature of lipids of inner leaflets.

## Positive Spontaneous Curvature of Inner Leaflets Promotes the Growth of Pores Induced by WT HA

We also showed that increasing the positive spontaneous curvature of inner leaflets promotes enlargement of pores formed by the intact WT HA. By keeping pH too high or temperature too low (i.e. "suboptimal" fusogenic conditions), we were able to induce lipid continuity, measured by spread of a fluorescent lipid dye from RBCs to HA-cells, without spread of aqueous dye loaded into the RBCs. We refer to this pattern of dye spread with HA-cells as "stunted fusion." This pattern could be due to hemifusion, or due to small pores that did not enlarge. We have shown by dye transfer experiments that stunted fusion is distinct from stable hemifusion: MPCAs promoted transfer of aqueous dye in stunted fusion with almost an order of magnitude lower concentration than was required for the same result with GPI-HA cells. We also showed that when MPCAs were added to GPI-HA cells that were hemifused to RBCs, more pores formed with increasing concentration of MPCA, but the average pore size did not increase. In contrast, for stunted fusion the average size of pores (perhaps formed by the MPCA) gradually enlarged as the concentration of MPCA was increased. These results indicate that pore growth is not only under control of the fusion protein, but under lipid control as well. The more positive the spontaneous curvature of the inner leaflet, the more readily a pore enlarges. Thus, these MPCA experiments combined with those using GPI-HA provide evidence for the concept that the two lipid leaflets perform different roles in the fusion process: outer leaflets are responsible for hemifusion; inner leaflets control pore formation and enlargement.

## References


Bullough, P.A., F.M. Hughson, J.J. Skehel, and D.C. Wiley. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371:37-43.

Chernomordik, L.V., M.M. Kozlov, and J. Zimmerberg. 1995. Lipids in biological membrane fusion. *J. Membr. Biol.* 146:1-14.

Gaudin, Y., R.W.H. Ruigrok, and J. Brunner. 1995. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. *J. Gen. Virol.* 76:1541-1556.

Günter-Ausborn, S., A. Praetor, and T. Stegmann. 1995. Inhibition of influenza-induced membrane fusion by lysophosphatidylcholine. *J. Biol. Chem.* 270:29279-29285.

Helfrich, W. 1973. Elastic properties of lipid bilayers: Theory and possible experiments. *Zeitschrift fur Naturforschung* 28:693-703.

Hernandez, L.D., L.R. Hoffrman, T.G. Wolfsberg, and J.M. White. 1996. Virus-cell and cell-cell fusion. *Annu. Rev. Cell Dev. Biol.* 12: 627-661.

Hornby, A.P. and P.R. Cullis. 1981. Influence of local and neutral anaesthetics on the polymorphic phase preferences of egg yolk phosphatidylethanolamine. *Biochim. Biophys. Acta* 647:285-292.

Jin, H., G.P. Leser, and R.A. Lamb. 1994. The influenza virus hemagglutinin cytoplasmic tail is not essential for virus assembly or infectivity. *EMBO J.* 13: 5504-5515.

Jin, H., K. Subbarao, S. Bagai, G.P. Leser, B.R. Murphy, and R.A. Lamb. 1996. Palmitylation of influenza virus hemagglutinin (H3) is not essential for virus assembly or infectivity. *J. Virol.* 70:1406-1414.

Kemble, G.W., Y.I. Henis, J.M. White. 1993. GPI- and Transmembrane-Anchored Influenza Hemagglutinin Differ in Structure and Receptor Binding Activity. *J. Cell Biol.* 122:1253-1265.

Kemble, G.W., T. Danieli, and J.M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* 76:383-391.

Kozlov, M.M., S.L. Leikin, L.V. Chernomordik, V.S. Markin, Yu.A. Chizmadzhev. 1989. Stalk mechanism of membrane fusion. *Eur. Biophys. J.* 17:121-129.

Melikyan, G.B., J.M. White, and F.S. Cohen. 1995. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* 131:679-691.

Melikyan, G.B., S.A. Brener, D.C. Ok, and F.S. Cohen. 1997a. Inner, but outer, membrane leaflets control the transition from glycosylphosphatidylinosiiol-anchored influenza hemagglutinin-induced hemifusion to full fusion. *J. Cell Biol.* 136:995-1005.

Melikyan, G.B., H. Jin, R.A. Lamb, and F.S. Cohen. 1997b. The role of the cytoplasmic tail region of HA in formation and growth of fusion pores. *Virology* 235:118-128.

Palade, G.E. 1975. Intracellular aspects of the process of protein synthesis. *Science* 189: 347-358.

Simpson, D.A. and R.A. Lamb. 1992. Alterations to influenza virus hemagglutinin cytoplasmic tail modulate virus infectivity. *J. Virol.* 66:790-803.

Shangguan T.D., D. Alford, and J. Bentz. 1996. Influenza virus-liposome lipid mixing is leaky and largely insensitive to the material properites of the target membrane. *Biochemistry* 35:4956-4965.

Sheetz, M.P. and S.J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA* 71:4457-4461.

Steck, T.L. 1989. Red cell shape. *In:* Cell Shape: Determinants, Regulation and Regulatory Role, pgs. 205-246. Eds. Stein, W.D. and Bronner, F. Academic Press, Inc., San Diego, CA.

Wiley, D.C. and J.J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Ann. Rev. Biochem.* 56:365-394.

Wilson, I.A., J.J. Skehel, and D.C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289:366-373.