Mechanism of Membrane Fusion Mediated with Viral Proteins

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ABSTRACT. The study of membrane fusion is an important subject of cell biology, especially intracellular transport system, which works through membrane fusion of transport vesicles. Nevertheless we have few informations about cellular proteins responsible for fusion reaction. Study of fusion mechanism has been done mainly with viral fusion-active proteins. The most intensively studied fusion-active protein is hemagglutinin (HA) of influenza A virus, and it has been shown that fusion-active subunit of HA is transformed into a long α-helical coiled coil at low pH and bridges two adjacent membranes with two hydrophobic regions, namely the fusion peptide region and the anchor region. Flexible bridge of the coiled coil may mediate mixing of the lipid bilayers, eventually resulting in membrane fusion. In this review, we focus on the molecular mechanism of membrane fusion, which has been elucidated mainly through the studies with influenza HA protein.

Key words : membrane fusion — hemifusion — enveloped virus — F protein — hemagglutinin — influenza virus

Membrane fusion activity is essential for enveloped viruses to initiate viral infection. Through the fusion of viral envelope with cellular membrane the virus delivers its genome into the cytoplasm of host cell. Membrane fusion is an important subject not only in the field of virology but also in the field of cell biology, especially intracellular transport system, which is driven by membrane fusion of transport vesicles. Although the study of membrane fusion is a general subject of biology, we have only few informations about cellular protein responsible for membrane fusion, and the study has been done mainly with viral fusion-active proteins.

Fusion-active protein was identified at first in Sendai virus by Homma and Ohuchi at1973.[1] The fusion (F) protein of Sendai virus is synthesized as a fusion-inactive precursor and then activated by proteolytic cleavage with protease such as trypsin.[2,3] Till now, many fusion-active proteins have been identified in various viruses. In most cases, they are synthesized as fusion-inactive precursors and then cleaved into fusion-active subunits by cellular protease(s). Fusion-active viral proteins can be classified into two groups according to their optimal pH for fusion reaction. One of them promotes membrane fusion at neutral pH and thereby the viral genome enters cytoplasm through the cytoplasmic membrane (Fig 1A). The other mediates

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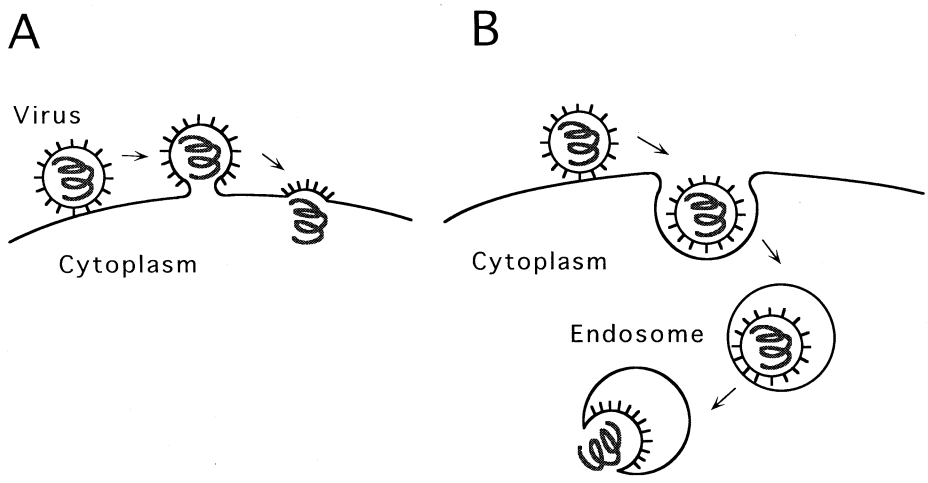

Fig 1. Entry pathway of enveloped viruses by membrane fusion. A) Viral envelope fuses with the cytoplasmic membrane. B) Viral envelope fuses with the endosomal membrane under acidic condition in endosome.

membrane fusion at acidic pH and thereby the viral genome enters cytoplasm through the endosomal membrane under the acidic conditions in endosome (Fig 1B). F protein of Sendai virus belongs to the former group and hemagglutinin (HA) of influenza A virus belongs to the latter group.[4,5] HA is the most intensively studied fusion-active protein. This is owing to the fact that HA is the only fusion-active protein, of which the three-dimensional structure has been clarified through X-ray crystallography.[6] In this review, we focus on the molecular mechanism of membrane fusion, which has been mainly studied with influenza HA protein.

CLEAVAGE ACTIVATION OF HA

Proteolytic cleavage of HA into subunits HA1 and HA2 is prerequisite[7,8] for membrane fusion activity and the cleaved HA mediates fusion reaction under acidic conditions.[9] As shown in Fig 2,[10] the cleavage site of HAs of human influenza viruses (subtypes H1 to H3) and avian apathogenic influenza viruses (subtypes H4, H6 and H8 to H13) consists of single arginine, which can be cleaved only by limited proteases such as Clara protease in lung[11] and trypsin. It means that these viruses can not infect cells in the absence of activating protease and therefore the infection is restricted in limited regions where the appropriate activating enzyme is present. On the other hand, HAs of avian highly pathogenic influenza viruses (subtypes H5 and H7) contains successive basic amino acids at the cleavage site,[12] which can be easily cleaved by ubiquitous intracellular proteases such as furin.[13,14] In this case, every virus carries already activated HA, and therefore such virus may cause pantropic systemic infection. Thus the cleavability of HA is an important determinant of pathogenisity of influenza virus.[15–17] This is the case also with paramyxoviruses.[18–20]

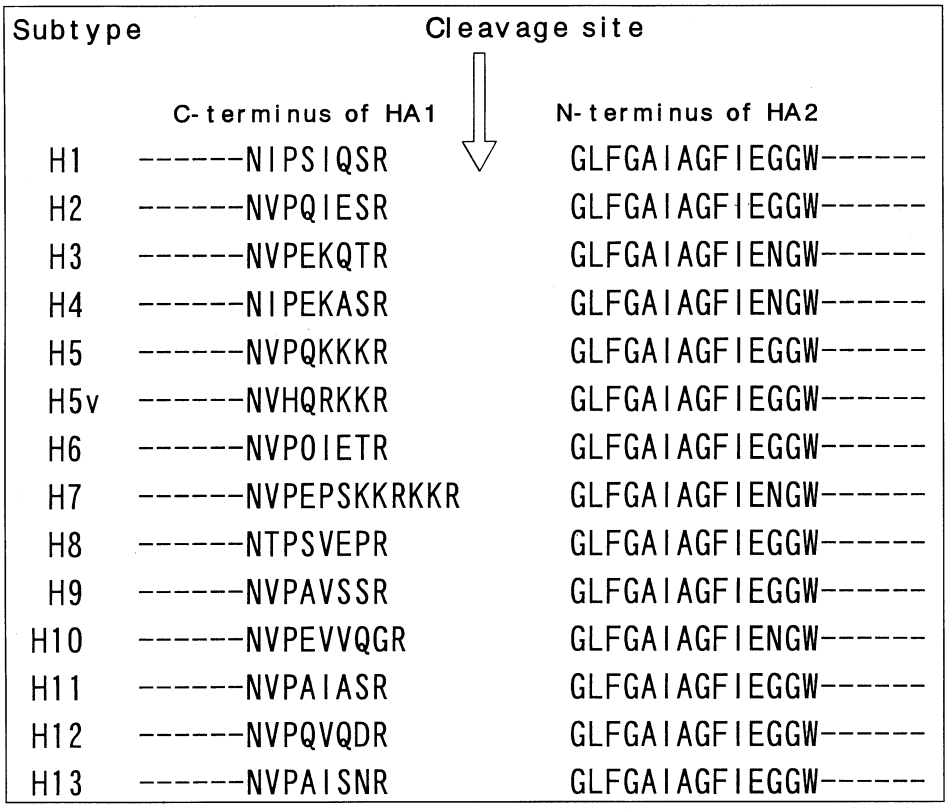

Fig 2. Cleavage sites of hemagglutinin (HA) of various subtypes of influenza virus. H5v is a variant of H5 subtype.

ACTION OF FUSION PEPTIDE

It is generally accepted that the fusion activity of HA is mediated by the hydrophobic region at the amino terminus of the HA2 subunit.[9] This region is called "fusion peptide" and its amino acid sequence is strictly conserved among various influenza virus strains (Fig 2). At neutral pH the fusion peptide is located at the bottom of the stem region of HA spike.[6] An HA spike is composed of three HA polypeptides, namely homotrimer. The location of the fusion peptide is shown in the three-dimensional structure model of HA monomer (Fig 3A and B). In the HA spike, the fusion peptide is buried inside three stems of HA trimer (Fig 3C). It has been known that at low pH a conformational change of the ectodomain of HA occurs and the fusion peptide is inserted into the target membrane.[21–23] As the carboxyl terminus of HA2 is composed of a transmembrane anchor domain and a short cytoplasmic domain, HA2 should bridge two membranes with the amino terminus, namely the fusion peptide, and with the carboxyl terminus, namely the anchor domain (Fig 4A, B and C). This bridging may be necessary but not sufficient for membrane fusion. Deletion or subsitution of even one amino acid in the fusion peptide abolished completely the fusion activity, although the deletion or substitution mutant retained the activity to plunge the fusion peptide into the target

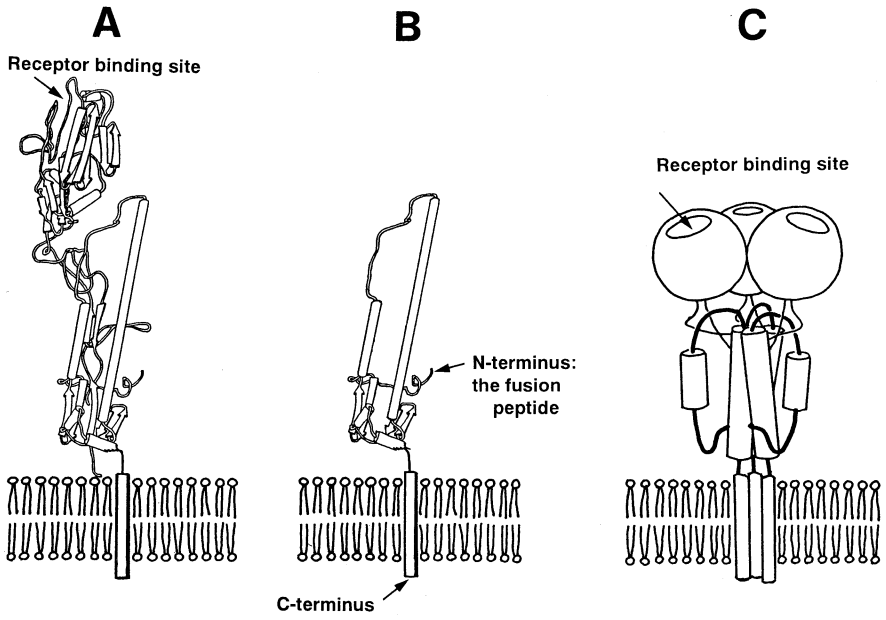

Fig 3. Three-dimensional structure of HA spike. A) Monomer of HA. The model of Wilson *et al*[6] was modified by adding the transmembrane and cytoplasmic domains. B) HA2 monomer, with HA1 domain omitted for clarity. C) Schematic model of HA spike (an HA spike is the trimer of HA polypeptides). The Model of Yu *et al*[23] was modified by adding the heads (HA1 region).

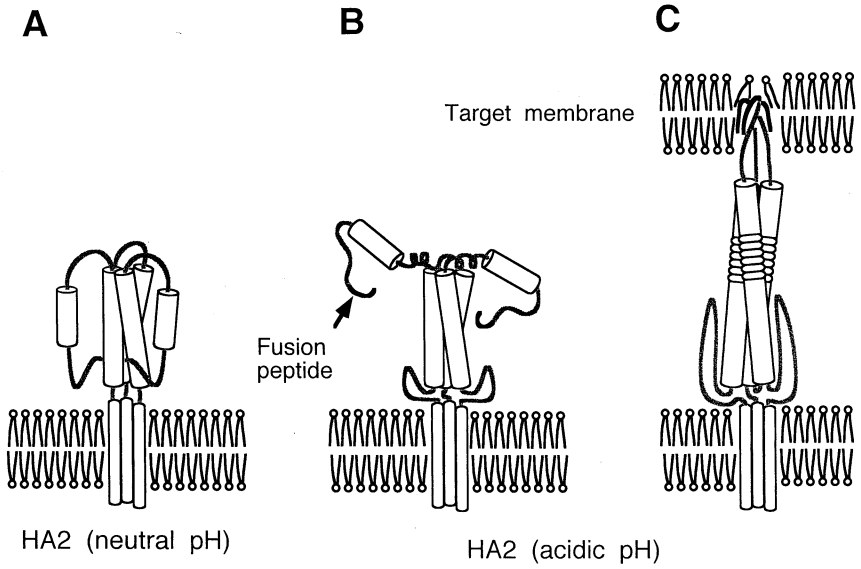

Fig 4. Conformational change of HA2 subunit and insertion of the fusion peptide into the target membrane. A) HA2 region at neutral pH. B) Beginning of conformational change of HA2 region. The loop region begins to be folded in α-helix and the fusion peptide appears outside. C) The fusion peptide is inserted into the target membrane.

membrane.[24,25] This observation indicates that not only hydrophobicity but also some special structure is required for the fusion peptide to mediate membrane fusion. Very similar sequence as the fusion peptide is observed at the amino termini of F1 which are generated by proteolytic cleavage of F proteins of paramyxoviruses.[26] Asano *et al* showed that the amino terminus of Sendai virus F1 recognized the structure of cholesterol and its derivatives, which are known as important components of membrane. They thought that the binding of F1 to cholesterol is essential for membrane fusion.[27] Such receptor-like substance has not yet been identified with the fusion peptide of influenza HA.

HOW CAN THE FUSION PEPTIDE APPROACH THE TARGET MEMBRANE?

As mentioned above the fusion peptide is located distant from the membrane of the target cell at neutral pH. Between the target membrane and the fusion peptide there are head region and long stem region (Fig 3C). Three heads carrying receptor binding activity must be removed or relocated to allow the fusion peptide to interact with the target membrane. Godley *et al* showed that introduction of intermolecular disulfide bondages which connect the heads each other abolished the fusion activity completely and the activity was restored after reductive agent treatment which should release the bondages.[28] Thus, the opening of the heads is proven to be prerequisite for membrane fusion (Fig 5). However, it is clear that the opening is not sufficient for the fusion peptide to approach the target membrane. The fusion peptide is located at the bottom of the longest α-helix in HA molecule. The fusion peptide is connected with a short α-helix which links with a loop and then the top of the longest α-helix (Fig 3B). To explain how the fusion peptide can approach the target membrane, Carr and Kim have proposed the spring-loaded model.[29] They analyzed animo acid sequence of the loop connecting the short α-helix

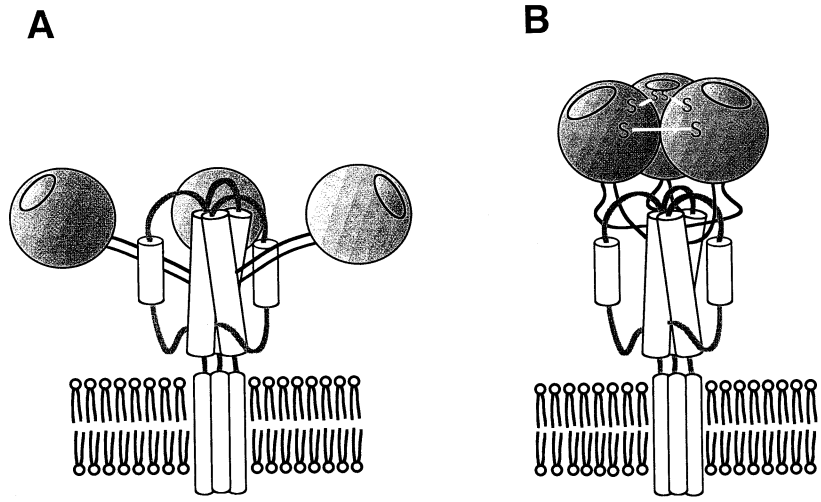

Fig 5. Opening the head region at low pH is necessary for fusion activity. A) Wild type HA. The head region opens at low pH. B) Mutant HA, in which intermolecular disulfide bonds were introduced. Opening the head is impossible unless the intermolecular disulfide bond is reduced.

and the longest α-helix and found that this loop has a potential to form α-helix. They synthesized artificially a peptide corresponding to the entire loop region plus part of the longest α-helix and found that this peptide was a random coil at neutral pH but formed trimer of α-helix at the low pH which could convert HA fusion-active. If this is also the case in HA molecule, the loop region would be folded at low pH and consequently form a further longer α-helix coiled coil (Fig 4B and C). Through such conformational change, the fusion peptide could move 100 Å toward the target membrane. Bullough *et al* analyzed the soluble part of HA2 by X-ray crystallography and found that the loop was folded and formed the α-helix coiled coil.[30] They thought also that the fusion peptide could move 100 Å toward the tip of HA molecule through the formation of the long α-helix coiled coil. According to this model, the fusion peptide can easily approach the target membrane at low pH.

HA IS IN METASTABLE STATE

Carr and Kim synthesized a longer peptide which contained the sequence of entire short α-helix and loop region plus part of the longest α-helix, and found that the longer peptide formed trimer of α-helix (coiled coil) not only at low pH but also at neutral pH.[28] This finding indicates that the region composed of the short α-helix, the loop and the longest α-helix would rather form a further longer α-helix coiled coil if there were no structural constraint from other components surrounding this region. It means that this region should take a constrained structure in the HA spike at neutral pH, in other words, HA should be in metastable state at neutral pH and the acidification may release the tension included in HA spike through the conformational change. This concept is supported also by Chen *et al*[31] When cDNA coding a soluble part of HA2 was expressed in E. coli, they found that the soluble domain of HA2 took the low-pH-induced conformation even at neutral pH. This demonstrates again that the acidic form, namely the coiled coil structure, is energetically the most stable form if any other structural constraint is not present. Thus, when the head region of HA spike opens at low pH, only a small activating energy may be required to convert HA2 from metastable form into fusion-active form.

In metastable HA spike the fusion peptides are located at the bottom of HA spike and stay inside the trimer of long α-helix. This region is surrounded nine oligosaccharide side chains and these glycosylation sites are strictly conserved among all influenza A viruses (Fig 6A). Recently we demonstrated that these oligosaccharides in the stem region maintain HA in the metastable form required for fusion activity.[32] Additionally some Influenza viruses utilize also their ion channel protein M2 for maintaining HA in the metastable form.[33,34]

ROLE OF TRANSMEMBRANE AND CYTOPLASMIC DOMAINS OF HA IN FUSION REACTION

In contrast to the ectodomain of HA, structure-function relationships are less well understood in the transmembrane domain and in the cytoplasmic tail of HA. It is not clear how these regions contribute to membrane fusion. The

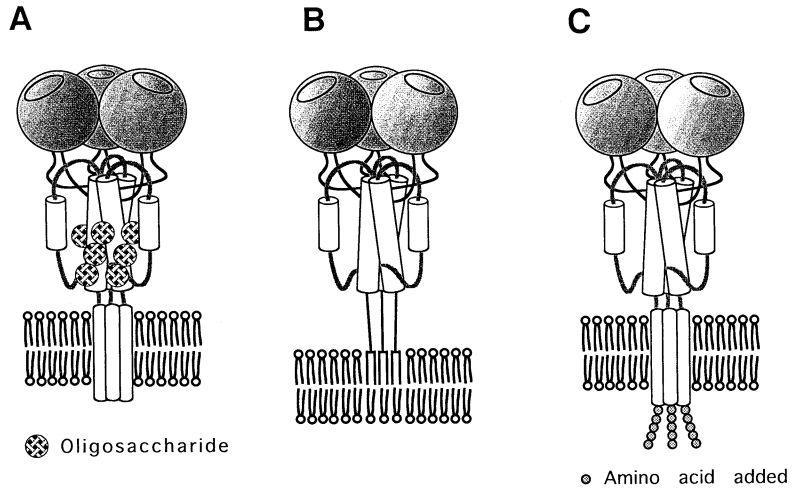

Fig 6. Conserved oligosaccharides at the stem of HA and variant HAs. A) The stem of HA spike is surrounded with nine oligosaccharide side chains. B) The transmembrane and cytoplasmic domains were exchanged for glycosylphophatidylinositol (GPI). C) Five amino acids were added to the end of the cytoplasmic tail.

first evidence for their involvement in fusion activity was obtained from the observation showing that deletion of the covalently bound fatty acids from the transmembrane and cytoplasmic domains of a subtype H2 HA abolished fusion activity.[35] This indicated that acylation of these domains was essential for fusion activity. In contrast, several other studies employing H2 and different HA subtypes showed fusion activity even after deletion of all fatty acids.[36–40]

Kemble *et al* substituted the glycosylphosphatidylinositol (GPI) anchor for the transmembrane and cytoplasmic domains of HA (Fig 6B) and found that the GPI-anchored HA mediated fusion of the outer leaflet of bilayer membrane but was not able to mediate full process of membrane fusion.[41] They called this step "hemifusion" (Fig 7). From this result they thought that not only the fusion peptide but also the transmembrane domain is required for HA to mediate the membrane fusion. Since it is known that the cytoplasmic domain of HA is not essential for virus infectivity,[42] they focused on the transmembrane domain as the region responsible for fusion activity. However, GPI-anchored HA differed in oligosaccharide processing from the original HA[43], indicating an abnormal folding of the ectodomain in GPI-anchored HA. It is therefore not clear whether the effect of GPI anchor on fusion activity is due to the loss of transmembrane domain or to the abnormal folding of the ectodomain in GPI-anchored HA.

Recently we found that elongation of the cytoplasmic tail (Fig 6C) reduced drastically fusion activity while it did not affect oligosaccharide processing, surface transport and receptor binding activity of HA.[44] The elongation of the tail interfered with formation and enlargement of fusion pores but not so with hemifusion. The interfering effect depended on the size, but not on the sequence of the attached peptides. The similar phenomenon has been reported with envelope glycoproteins of retroviruses including human and simian immunodefficiency viruses (HIV and SIV), that is, truncation at the carboxyl

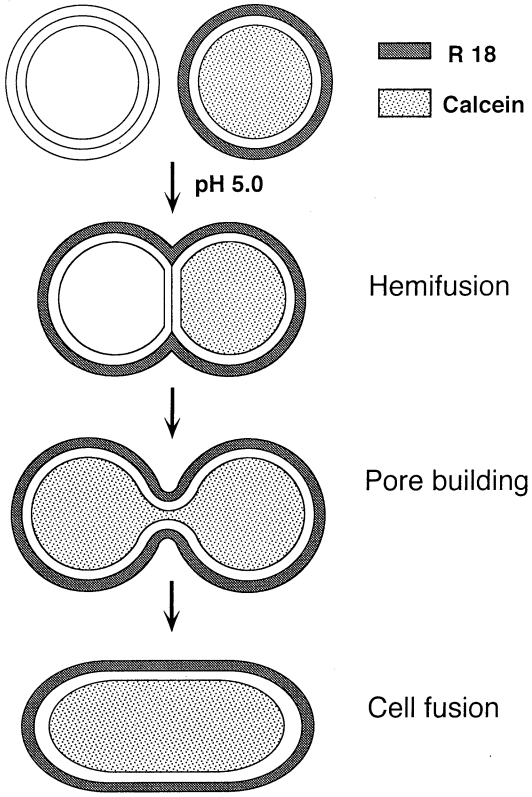

Fig 7. Fusion process. In hemifusion only the outer leaflets of lipid bilayer membrane are fused, and in fusion pore building process the soluble contents of cells are mixed. These processes can be monitored individually by labelling the outer leaflet of lopid bilayer with octadecylrhodamine (R18) or labelling the cell contents with calcein.

terminal end resulted in increased syncytium forming ability.[45–52]

Insight into the mechanism how the cytoplasmic tail modulates fusion comes from recent X-ray crystallographic studies performed on the acidic form of HA2, namely the fusion-active conformation,[8] and on the transmembrane subunits of envelope glycoproteins of murine leukemia virus[53] and HIV[54] that suggest a common mechanism for fusion initiation. According to these studies the core of the proteins is an extended, triple-stranded α-helical coiled coil, with the top ends of which the fusion peptides link. In gp 41, the transmembrane subunit of HIV, the coiled coil is surrounded with three other α-helices in the reverse direction.[54] This conformation places the amino-terminal fusion peptide and the carboxyl-terminal membrane anchor near each other (Fig 8). With the anchor embedded in the viral envelope and the fusion peptide inserted into the target membrane, this structure forces both membranes into close apposition. Electron microscopy and antibody labeling of membrane-associated HA2 provided further support for such structure.[55] Flexible links between the central rod, the fusion peptide, and the anchor may

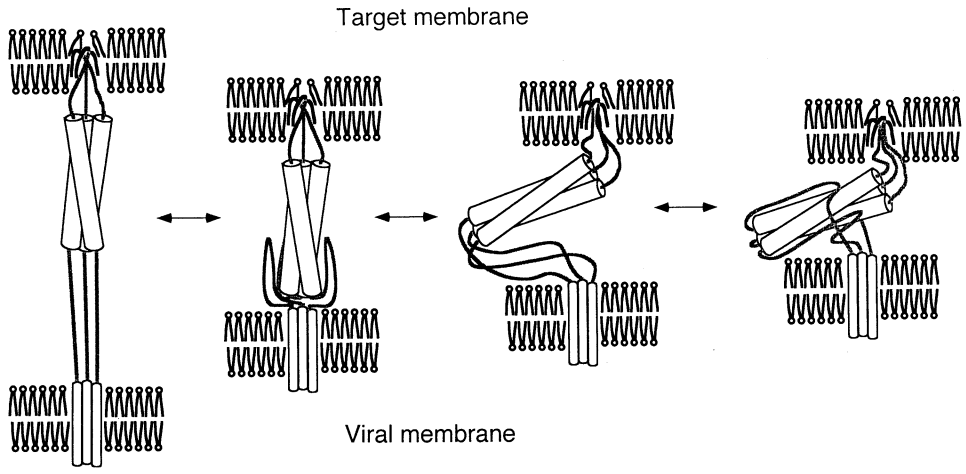

Fig 8. Flexible bridge of HA2 or gp41. The flexible links between the central rod and the fusion peptide and the anchor may allow oscillation of both hydrophobic domains in the adjacent membranes which may cause perturbation and mixing of the lipid bilayers, eventually resulting in fusion.

allow oscillation of both hydrophobic domains in the adjacent membranes which may cause perturbation and mixing of the lipid bilayers, eventually resulting in fusion.[25,54] It is an intrinsic feature of this model that fusion peptide and membrane anchor have very similar functions in fusion, and the observation that the process proceeds only to hemifusion when the anchor peptide is replaced with GPI lipid anchor indicates that both hydrophobic peptide domains are equally important for complete fusion.[41] On the other hand, the mobility of the membrane anchor may be limited by the cytoplasmic tail and this constraint may interfere with fusion when the tail is elongated beyond its natural size.[44] Restriction of anchor mobility by an elongated tail should therefore have the same effect as replacement of the transmembrane peptide with GPI anchor, i.e. it should interfere with formation and enlargement of fusion pores rather than with hemifusion.

Studies with acylation mutants of FPV HA indicated that loss of the tail fatty acids reduces fusion activity similar to tail elongation (manuscript in preparation). Thus, it appears that size and hydrophobicity of the tail are important determinants for anchor mobility. Our observation that removal of the tail has only small effect on fusion[44] is conform with this concept. These data are also compatible with other models proposed by Blumenthal *et al*[56] and Kanaseki *et al*.[57] The former group analyzed the kinetics of fusion pore formation and the latter group succeeded in direct observation of fusion pores through the quick-freezing electron microscopy.

CONCLUSION

Molecular mechanism of membrane fusion is not yet fully understood, but, as mentioned above, a lot of informations have been accumulated enough to allow us speculate the mechanism. Two adjacent membranes should be bridged

with the long α-helical coiled coil. The top end of the coiled coil has the fusion peptide inserted into the target membrane and the other end of the coil links with the anchor domain embedded in the viral envelope or in the membrane of virus-infected cell. The bridge may be folded reversibly at the junction of the coiled coil and the anchor domain. This folding may cause perturbation and mixing of the lipid bilayers, eventually resulting in membrane fusion.

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