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Review

Membrane fusion: the process and its energy suppliers

G. Basañez

Unidad de Biofísica (CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, P.O. Box 644, 48080 Bilbao (Spain), Fax + 34 94 464 8500, e-mail: gbzbaasg@lg.ehu.es

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Abstract. Membrane fusion constitutes a pivotal process in eukaryotic cell physiology. Both specialized proteins and membrane lipids play key roles in fusion. Here, our current understanding of the mechanism of membrane fusion is reviewed. The focus is on the relatively simple and well-understood proteinaceous fusion machinery of enveloped viruses and the physical properties of lipids that appear to be of great relevance for fusion progression. Recent observations suggest that viral fusion proteins use packed conformational energy and bilayer-destabilizing domains to (i) bring participating membranes into intimate contact, (ii) merge proximal lipid monolayers through highly curved stalk/hemifusion intermediates, and (iii) generate a lipid-containing fusion pore, thereby terminating the fusion process.

Key words. Membrane fusion; lipid polymorphism; membrane curvature; viral glycoprotein; influenza hemagglutinin; HIV-1 gp41; viral fusion peptide.

Introduction

Membrane fusion is fundamental for eukaryotic life. A plethora of biological phenomena including sexual reproduction, the formation of multinucleated cells, intercellular communication, and intracellular trafficking and recycling of membranes all involve this essential process [1, 2]. Cellular invasion by enveloped pathogenic viruses such as influenza and HIV also requires merging of viral and cellular membranes [3, 4]. In addition, membrane-coated vehicles that mimic viral entry into cells have gained increased interest as targeted drug and intracellular gene delivery systems [5]. Deciphering the molecular mechanism of fusion, consequently, poses an outstanding challenge to scientists, not only because of its physiological relevance but also because of its interest for medical and technological purposes.

Biomembrane fusion is a complex process tightly regulated by specialized proteins. However, distinguishing between proteins directly involved in membrane fusion itself and proteins implicated in processes preceding or following actual membrane coalescence has proven extremely difficult [6]. So far, viral surface glycoproteins are the only proteins unequivocally identified as being both necessary and sufficient for execution of biological membrane fusion. Although proteins are clearly essential to control the specific location, timing, and speed of fusion, at the same time, lipids are also active partners in this process since, ultimately, the lipidic part of the membranes must fuse [6, 7].

Since membrane fusion does not occur spontaneously under most physiologically relevant conditions, specialized proteins are often referred to as ‘catalysts’ of the fusion process. However, the physiological fusion process exhibits two main differences compared to classical enzymatic reactions. First, a single fusion act involves transient changes in the organization of hundreds of lipid molecules. Consequently, the height of the energy barrier to be overcome in the fusion process depends not only on specific characteristics of individual lipid molecules, but

* Corresponding author.

also on collective properties of membrane lipids. Second, the best understood fusion proteins seem to provide the free energy required to overcome the energy barrier for lipid bilayer fusion by transiting from a metastable state into a thermodynamically more favorable state, i.e., by undergoing an irreversible conformational change. Therefore, fusion proteins are best considered as local suppliers of energy, rather than as catalytic agents (enzymes).

The process: lipid bilayer merging

The fusion of two membranes in an aqueous environment implies that two separate lipid bilayers merge to become coplanar and mix the aqueous volumes that each of them initially encloses, without substantial spillage to the external medium. The presence of protein is not an absolute prerequisite for fusion: purely lipidic membranes can be induced to fuse in response to a variety of stimuli. Accumulating evidence indicates that the ultimate physicochemical mechanism underlying biomembrane fusion is very similar to that governing pure lipid fusion, and involves a common set of intermediate stages, in which membrane lipids momentarily and locally leave the planar bilayer architecture to adopt highly curved nonbilayer arrangements.

Lipid polymorphism

In addition to the bilayer (lamellar) phase broadly recognized as the dominant structural motif for biomembranes, lipids can adopt a wide variety of nonbilayer (nonlamellar) morphologies typically possessing spherical, hexagonal or cubic geometry [8, 9]. The different morphologies adopted by lipids can be rationalized according to the depth-dependent distribution of lateral pressures within a lipid monolayer [10] (fig. 1 A). In a planar monolayer at equilibrium, a uniform lateral pressure is exerted all along the lipid molecule (fig. 1 A-1). Any imbalance in the lateral pressure distribution will induce a tendency in the lipid monolayer to curl away from a planar, lamellar configuration into a curved, nonlamellar configuration. If a positive lateral pressure exists at the polar part of the lipid and a negative lateral pressure exists at the apolar part, formation of normal (type I) nonlamellar phases will be promoted in which acyl chains are directed toward the interior of the structure and headgroups are splayed outwards (fig. 1 A-2). Conversely, if the lateral pressure at the hydrophobic tail region dominates, inverted (type II) nonlamellar phases will be favored in which headgroups are facing toward a central aqueous channel and chains are directed outwards (fig. 1 A-3). Prominent examples of inverted nonlamellar phases are the hexagonal H_{II} and bicontinuous cubic Q_{II} phases (fig. 1 A-3, B). By convention, a lipid monolayer in a type I phase possesses positive mean interfacial curvature, whereas a lipid monolayer in a type II phase possesses negative mean interfacial curvature (a lipid monolayer in a lamellar phase, at equilibrium, possesses zero mean interfacial curvature). The polymorphic behavior of an individual lipid can also be rationalized by considering its effective molecular shape, which, in turn, is primarily determined by the relative surface areas occupied by the polar headgroup and the apolar hydrocarbon chains [8–11]. Important lipid components of eukaryotic membranes such as the zwitterionic phospholipids phosphatidylcholine (PC) and sphingomyelin (SM), and the negatively charged phospholipids phosphatidylserine (PS), cardiolipin (CL), and phosphatidic acid (PA), all have similar cross-sectional areas at the headgroup and hydrocarbon regions, under most physiological conditions. These lipids approximate cylinders in shape and form stable bilayers (lamellar-preferring lipids; fig. 1 A-1). On the other hand, biomem-

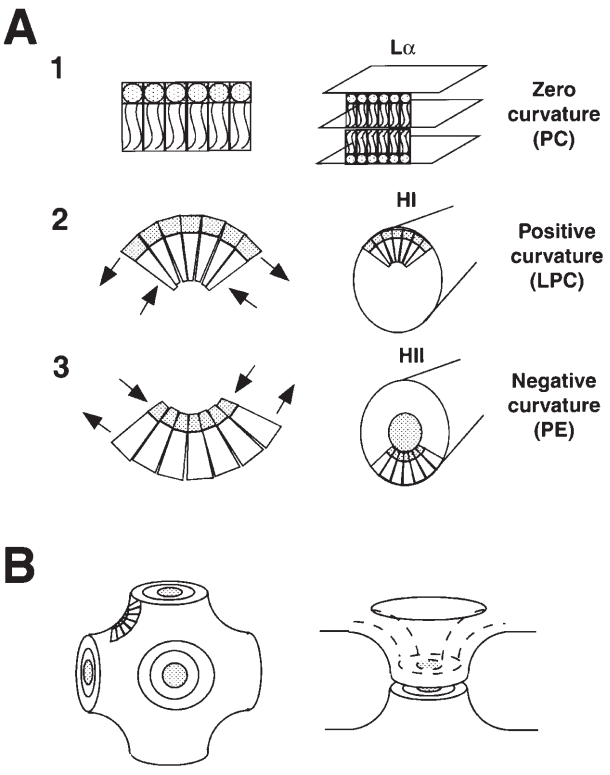

Figure 1. Lipid polymorphism. (*A*) Schematic representations of pure lipid monolayers composed of molecules with cylindrical (1), inverted conical (2) and conical (3) effective shapes. Left column: patterns of lateral forces exerted at the lipid headgroup and tail regions. Middle column: illustrative lipid phases formed at equilibrium. L, lamellar (bilayer) phase; HI, normal hexagonal phase; HII, inverted hexagonal phase. Right column: Corresponding mean interfacial curvatures, and representative lipid species (in parentheses) (*B*) Structural element of an inverted bicontinuous cubic phase QII (left), and its proposed correlation with a lipidic fusion pore (right). See text for more details.

brane lipids possessing a relatively large area at the headgroup in relation to the chain region exemplified by lysophosphatidylcholine (LPC) are best modeled as inverted cones, and form normal nonlamellar phases (type I lipids; fig. 1 A-2). Finally, physiologically relevant amphiphiles with a large apolar portion relative to the polar one, such as unsaturated species of phosphatidylethanolamine (PE), diacylglycerol (DAG), and fatty acid (FA), emulate cone-like shapes, and form, or promote formation of inverted nonlamellar phases (type II lipids; fig. 1 A-3).

The effective molecular shape of an individual lipid governs the intrinsic tendency of the monolayer to bend, i. e., the spontaneous curvature of the lipid monolayer in the stress-free state [12]. From this perspective, lipids with cylindrical, inverted-conical, and conical molecular shapes are said to possess zero, positive, and negative spontaneous curvature, respectively. However, the phase preference or spontaneous curvature of a particular lipid depends not only on the chemical structure of the molecule, but also on environmental factors and intra- and intermolecular interactions. For example, binding of Ca^{2+} ions to CL or PA can induce a closer packing of the headgroups, reducing the area subtended by the polar part of the lipid and thereby triggering a transition from a lamellar phase into a type II phase [13].

Nonlamellar lipids and membrane fusion

Multiple lines of evidence indicate that nonlamellar lipids can influence the outcome of the fusion reaction. Endocytic vesicles [14], secretory granules [15] and enveloped viruses [16–18] are all more prone to merge with pure lipid vesicles (liposomes) containing PE, compared to liposomes composed exclusively of lamellar-preferring lipids such as PC or SM. Likewise, the presence of PE seems to be required for liposomes to mimic the high fusogenicity of natural membranes [19, 20], and PE is commonly utilized in lipid-based intracellular drug delivery systems [5]. Similarly to PE, membrane-incorporated FA is also known to promote exocytosis [21, 22], organelle-organelle fusion [23, 24], viral fusion [25–27], and liposome-liposome fusion [28–30]. DAG greatly stimulates fusion efficiency in a number of biological and model membrane systems as well [31, and references therein]. However, not every exogenous nonlamellar lipid promotes fusion. LPC, for example, is a near-universal fusion inhibitor [32]. Other type I amphiphiles have also been found to inhibit diverse membrane fusion processes [33–40]. In summary, a general trend exists in exogenous type II lipids to promote membrane fusion, and in exogenous type I lipids to inhibit membrane fusion.

Lamellar lipids can be transformed into nonlamellar lipids in vivo by the catalytic activity of lipid-modifying enzymes, such as phospholipases. The most common phospholipases cleave intact phospholipids leaving DAG (phospholipase C), PA (phospholipase D), and FA and lysolipids (phospholipase A_2) as lipid catabolites in the membrane. In addition to their well-known function as signaling second messengers, phospholipase-generated products have been directly implicated in membrane-fusing events [41–50]. Thus, not only exogenous incorporation, but also in situ generation of nonlamellar lipids affects membrane fusion susceptibility.

How do nonlamellar lipids influence the fusion process? The fact that nonlamellar lipids affect biomembrane fusion and pure lipid fusion in a similar fashion, together with the absence of any specific chemical moiety directly correlating with fusion promotion or inhibition, suggests that nonlamellar lipids act by changing one (or several) physical parameter(s) of the lipid membrane matrix. A property of the membrane of great relevance for fusion is the spontaneous curvature of its constituent monolayers [32]. As discussed before, incorporation of any nonlamellar lipid in a planar monolayer will impart a desire for interfacial curvature, due to unequal distribution of lateral pressure between amphiphile headgroup and chain areas. However, in a bilayer membrane structure, monolayer bending is opposed by cohesive hydrophobic interactions acting against the peeling apart of the two leaflets. If, as a result of such competing forces, the spontaneous curvature of the nonlamellar lipid is not expressed, a curvature stress builds up in the bilayer [12, 51]. Such curvature stress can be described as elastic energy stored in the membrane with the latent ability to destabilize the lipid bilayer structure [52]. Therefore, nonlamellar lipids may modulate fusion by inducing curvature stress in the bilayer through changes in monolayer spontaneous curvature.

Stalk model of membrane fusion

Based on both the above-mentioned experimental findings and theoretical arguments, a specific fusion scheme known as the stalk hypothesis has been developing for more than two decades. Several versions of the stalk model have been proposed [32, 53–58], all sharing the following general outline: (i) establishment of *close contact* between participating lipid bilayers, (ii) merger of proximal (cis) lipid monolayers via *stalk*/*hemifusion* structures; and (iii) merger of distal (trans) lipid monolayers with concomitant opening of a *lipidic fusion pore* (fig. 2).

To fuse, the two membranes must first be brought into *close contact*. The main force opposing lipid bilayer approach at distances below ~2–3 nm is the repulsive hydration force [59]. Since this force is proportional to membrane area, less work is necessary if close approximation occurs at a localized point. However, merely surmounting the intermembrane hydration repulsion barrier

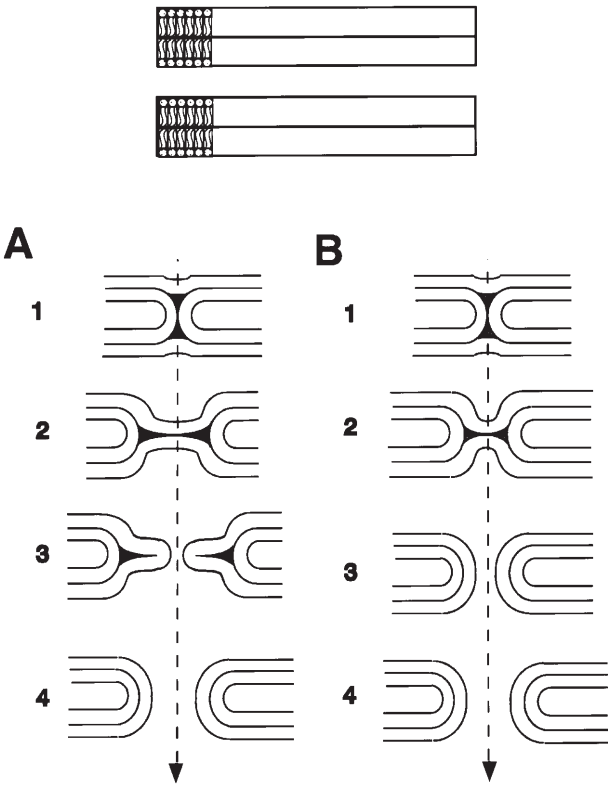

Figure 2. Stalk mechanisms of lipid bilayer fusion. Original stalk model (*A*) and modified stalk model (Siegel's version) (*B*). 1: stalk intermediate. Proximal leaflets of two interacting membranes merge, while distal leaflets are kept distant from each other. 2: hemifusion. Distal leaflets contact with each other to form a single bilayer that still impedes exchange of aqueous volumes. 3: initial lipidic fusion pore. A narrow aqueous connection is established that allows diffusion of small solutes (ions) across the membrane. 4: expanded lipidic fusion pore. Note the following: (i) lipid monolayers are considered flat initially (top), but acquire high curvature in fusion intermediates (1–3); (ii) to form nonlamellar stalk/hemifusion structures, the two monolayers of the original bilayer peel from one another creating energetically unfavorable hydrophobic interstices (voids) within fusing membranes (shaded areas in 1–3), (iii) the hemifusion zone in the original stalk model (A2) is large relative to that in the modified stalk model (B2); and (iv) initial fusion pores (3) are shaped differently in these two versions of the stalk hypothesis.

does not guarantee fusion; productive fusion also necessitates the building up of an attractive force between the hydrophobic interiors of closely juxtaposed membranes [60]. During biomembrane fusion, specific proteins, lipids, and/or lipid-modifying enzymes may participate in delimiting a focal point in the membrane for fusion progression, as well as in reducing the hydration repulsion barrier, and in perturbing the bilayer structure to create hydrophobic defects at the membrane surface.

Such hydrophobic patches are believed to act as nucleation sites for the first fusion intermediate: the *stalk* (fig. 2A-1). The stalk is depicted as a nonlamellar lipidic structure with an hourglass shape that connects only cis monolayers of interacting membranes. Although the stalk has not yet been directly demonstrated, this has been attributed to its very transient and localized nature. The stalk is then postulated to evolve into a *hemifusion* intermediate (fig. 2A-2). Here, trans monolayers touch each other forming a single bilayer that continues to separate the aqueous compartments. Experimentally, hemifusion is usually defined as mixing of membrane lipids without mixing of aqueous contents. Such an intermediate stage of membrane fusion has been demonstrated in a wide variety of model systems [61–67]. Hemifusion states have also been captured and extensively characterized in multiple viral-mediated membrane fusion events [68–79].

How do two hemifused membranes evolve toward their complete fusion? In its original formulation, the model proposed that the hemifusion zone expands radially due to relaxation of monolayer bending energy and/or aided by membrane surface tension, until an extended bilayer septum or hemifusion diaphragm is formed [32]. Membrane surface tension build-up would later rupture the hemifusion diaphragm-opening a *lipidic fusion pore*, i.e., a narrow passageway across the bilayer that connects previously separated aqueous compartments, and that is lined by trans monolayer lipid headgroups (fig. 2A-3). Experimental evidence supports the very small size of the initial fusion pore as well as its lipidic composition [80], although the latter issue is still a matter of discussion [81]. Lipidic fusion pores tend to open and close (flicker) before expanding irreversibly [62], thereby terminating the fusion process (fig. 2A-4).

Both the stalk and the lipidic fusion pore possess highly curved shapes. Therefore, the energy required to create such nonlamellar lipid structures should depend on spontaneous monolayer curvature. Considering two planar membranes brought into close proximity, theoretical estimates showed that the more negative the spontaneous curvature of cis monolayers, the more energetically favorable was stalk formation [61]. This prediction is strongly supported by the close to universal ability of exogenous type II nonlamellar lipids to promote fusion, and of exogenous type I nonlamellar lipids to inhibit fusion, as discussed before. On the other hand, also in light of theoretical calculations, the probability of lipidic fusion pore formation/expansion within the hemifusion diaphragm was predicted to be greater, the more positive the spontaneous curvature of trans monolayers [82]. A number of experimental observations are in agreement with this expectation as well [26, 61, 63, 83]. In short, according to the original stalk model, the willingness of a lipid bilayer to fuse relies upon the bending propensity of its constituent monolayers, with the most fusogenic membranes being those possessing negative and positive spontaneous curvatures in cis and trans monolayers, respectively.

Subsequent work by Siegel brought to attention factors other than monolayer curvature that may contribute to

evolution of fusion intermediates [54, 55, 84]. Siegel noted that stalk formation should be opposed by the energy required to create hydrophobic void spaces within fusing membranes, very much like lamellar-H_{II} phase transitions are thought to be opposed by the energy associated with formation of interstices between hexagonal cylinders of the H_{II} phase [54]. Such hydrophobic cavities are thought to preclude expansion of the hemifused zone, the stalk only evolving into a highly localized hemifusion structure or trans monolayer contact (TMC) (fig. 2B-2). The TMC intermediate would then reconfigure into a fusion pore or interlamellar attachment site (ILA) (fig. 2B-3), an event presumably governed by the bilayer rupture tension, a material property of the membrane which depends on its lipid composition [55, 84].

Interestingly, according to Siegel's calculations, fusion pore/ILA formation should be favored when trans monolayers possess negative spontaneous curvature [54], not positive spontaneous curvature as proposed in the original stalk model [82]. Additionally, this author posited that ILAs are key precursors of bicontinuous Q_{II} phases, and that the mechanism of formation of lipid fusion intermediates is similar to the mechanism of formation of bicontinuous Q_{II} phase precursors [55]. These considerations are consistent with the high fusogenicity of membrane systems with negative spontaneous curvature in *both* monolayers, and with the tendency of such lipid dispersions to form bicontinuous Q_{II} phases under equilibrium conditions [30, 84–92]. In summary, in this modified version of the stalk model, the fusion process relies upon the propensity of interacting lipid bilayers to form stalks, TMCs and ILAs, and progression through these putative fusion intermediates is energetically more favorable in the vicinity of a lamellar-bicontinuous Q_{II} phase transition.

One long-standing dilemma of the stalk model is the unrealistically high energy predicted for hypothetical intermediates along the fusion pathway. Recently, much effort has been devoted to solve this problem by considering additional monolayer deformations [56, 57], as well as alternative geometries of intermediate structures [58]. Both factors have been shown to reduce considerably the total energy of the fusion process [56–58]. Future experimental work may help to determine the applicability of these novel theoretical postulates to pure lipid and biological membrane fusion.

Viral fusion proteins: biological suppliers of fusogenic energy

To date, surface glycoproteins of enveloped viruses are the best-understood biological fusion protein machines. Viral fusion proteins have in common a number of features. (i) Viral fusion proteins undergo a maturation step that renders them metastable, primed for shifting into a more stable state upon receiving the appropriate fusion-triggering signal (low pH or receptor binding). (ii) In the fusogenic state, they are almost invariably trimeric, and aggregation of multiple trimers often helps fusion. (iii) Typically, each monomer within the oligomer can be conveniently divided into three segments: an extraviral part known as the ectodomain, a single transmembrane domain, and an intraviral segment or cytoplasmic tail. (iv) Upon fusion triggering, regions within the ectodomain acquire the competence to bind to and insert into membranes. Of special relevance is the fusion peptide: a relatively short, hydrophobic and well-conserved stretch of amino acids that is indispensable for fusion. (v) The fusion peptide-harboring ectodomain appears to be primarily responsible for initial steps of the fusion process, whereas the transmembrane domain seems to be fundamental for late steps of the fusion process. (The cytoplasmic tail is thought not to directly contribute to fusion.)

During the last decade, the core structures of many viral fusion proteins have been solved at the atomic level. However, for several reasons, the structure-function relationship in viral protein-mediated fusion is still not completely understood. First and foremost, influenza hemagglutinin (HA) is the only viral fusion protein for which detailed structural information is available in both its prefusogenic and fusion-activated conformations. Furthermore, most high-resolution structures obtained to date correspond to soluble fragments of viral fusion proteins; structural information of membrane-anchored entire viral fusion proteins is much more rare. Additional complications arise because crystallized viral fusion protein fragments are static structures of extraordinary homogeneity, while viral protein-mediated biomembrane fusion is a highly dynamic process in which only a fraction of the total protein population is actually engaged. Hence, correlating a particular equilibrium protein structure with a precise stage along the membrane fusion pathway is difficult.

With these caveats in mind, some ways through which viral fusion proteins may cause membrane fusion can be discussed. Particular emphasis will be given to putative roles played by distinct regions of viral fusion proteins in the overall fusion process. For more thorough overviews on viral membrane fusion, the reader is referred to several excellent recent reviews [1, 3–4, 93–95]

Soluble structural elements

Structurally, soluble parts of ectodomains of viral fusion proteins can be classified into two main groups [93]: those in which α helices are the prevalent secondary elements (class I viral fusion proteins) and those in which the polypeptide chain is almost exclusively folded as antiparallel β sheets (class II viral fusion proteins).

Helical bundles

Fusion proteins of orthomixo-, retro-, filo- and paramyxoviruses all contain [3, 4] (or are predicted to contain) [96] a characteristic ectodomain core structure consisting of a triple-stranded coiled-coil helical bundle. This type of structurally related, homotrimeric viral fusion protein is synthesized in an immature precursor form that is itself subsequently cleaved to generate a receptor-binding subunit and a fusogenic subunit, the latter possessing the fusion peptide at or near its NH_2 terminus. Two well-known class I viral fusion protein representatives are influenza HA and HIV-1 Env.

To enter a cell, influenza virus first binds to specific cell plasma membrane receptors, then becomes endocytosed, and, finally, upon acidification of the endosomal interior, fusion of viral and endosomal membranes ensues [1]. HA1 and HA2 subunits of HA are responsible for receptor binding and for low pH-mediated membrane fusion, respectively [3]. Structural and biochemical studies have shown that in neutral pH (native) HA, three HA1 subunits are positioned in the periphery of the molecule with their receptor-binding globular domains sitting atop, whereas three fusogenic HA2 subunits are positioned in the center of the molecule with their C termini anchored to the membrane (fig. 3 A, left) [1, 97]. Proceeding N terminally, the most relevant structural features of an HA2 monomer are a 29-residue-long α-helical region involved in trimeric coiled-coil formation, a 21-residue-long loop segment, a short helical region running antiparallel to the central coiled-coil, and the NH_2 terminal fusion peptide comprising the last 20–25 residues. Of note, in neutral pH native HA, the fusion peptide is hidden at the triple coiled-coil interface, inaccessible for membrane interaction.

Compared to the neutral pH crystal structure of HA, three major differences are observed in crystallized ectodomain core fragments of HA (lacking HA1 and the fusion peptide) treated with acid pH [98] or expressed in bacteria [99, 100] (fig. 3 A, right). First, what were a disordered loop and a short helix in neutral pH HA2 are recruited into the central coiled-coil in low pH-treated HA2. Second, 6 residues in the middle of the 29-residue-long α helix transform into a 180° turn, causing the polypeptide chain to reverse direction, and thereby packing the remainder of the helix antiparallel against the central coiled-coil. Third, the C-terminal portion of the ectodomain that had a compact structure in native HA2 adopts a mostly extended conformation at fusogenic pH, while continuing to proceed toward the N-terminal end of the structure. In the context of the whole HA bound to membrane, HA1 subunits are thought to dissociate to allow HA2 to adopt the above-described triple hairpin-like structure (also known as the six-helix bundle), with each identical hairpin possessing the membrane-embedded N-terminal fusion peptide and the C-terminal transmembrane domain in close proximity.

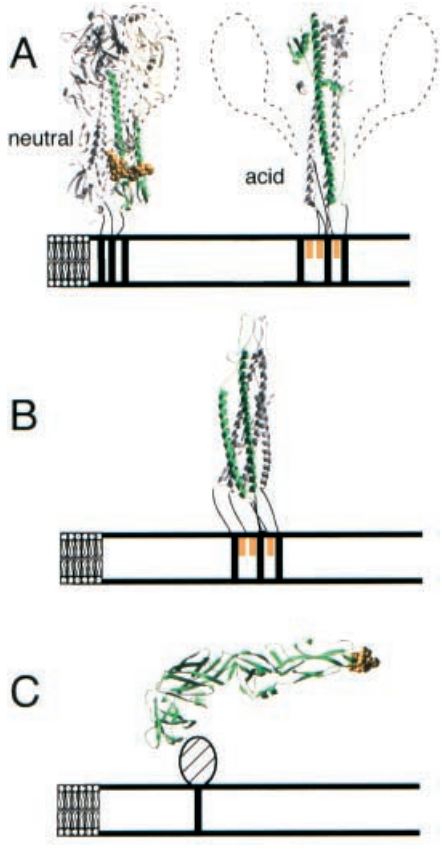

Figure 3. Structures of representative viral fusion proteins in their presumptive membrane-bound state: Neutral pH (native) and acid pH (fusion-activated) conformations of influenza HA [receptor-binding subunits (HA1) of the molecule are indicated encircled by dotted lines] (*A*); 'hairpin-trimer' (fusion-activated) conformation of HIV-1 gp41 (*B*); neutral pH (native) conformation of tick-borne encephalitis virus E protein (TBE-E). Only a single monomer of the TBE-E dimer is shown. The hatched ellipsoid represents the missing domain in the crystal (approximately 100 amino acids) (*C*). In all structures, monomers of the fusogenic subunit are colored in green, and ribbon representations highlight the principal elements of secondary structure. Orange balls in *A* and in *C* correspond to atomic Van der Waals surfaces of fusion peptides. Membrane regions: transmembrane anchors (black), and inserted fusion peptides (orange). Atomic structures, 2HMG (HA neutral), 1QU1 (HA2 acid) 1IF3 (HIV-1 gp41) and 1SVB (TBE-E) were rendered with Swiss-PDBviewer.

The fact that the ectodomain of HA2 expressed in bacteria without HA1 folds spontaneously into the same soluble core structure as the low pH-treated HA2 ectodomain gave rise to the idea that native HA exists in a metastable fold, in which HA1 prevents HA2 from achieving its most stable six-helix bundle fold [99]. Consistent with this interpretation, high temperature or other protein-destabilizing factors can replace low pH in activation of the conformational change in HA2 and in HA-mediated fusion [101–103]. In contrast, HA2 ectodomain core structures exposed to low pH or expressed by themselves in bacteria are highly thermostable [101, 102].

Unlike influenza, retroviruses enter the host cell through fusion with the plasma membrane at neutral pH [4]. This process is mediated by the retroviral Env viral surface glycoprotein consisting of a receptor-binding subunit and a membrane-anchored fusogenic subunit designated gp120 and gp41, respectively. Binding of gp120 to specific cell surface receptors triggers a cascade of conformational changes culminating in the activation of gp41, thereby leading to fusion of viral envelope and cellular plasma membranes. The solution structure of the ectodomain core of gp41 (in the absence of gp120 and the fusion peptide) also consists of a trimer-of-hairpins or six-stranded helical bundle: three N-terminal helices form a central triple-stranded coiled-coil, and are surrounded by three C-terminal helices that pack antiparallel within hydrophobic grooves on the surface of the coiled-coil core (fig. 3B) [104–107]. By analogy to HA2, this highly stable structure, presumably having the fusion peptide and the transmembrane domain located close together within the membrane milieu, has been proposed to represent the low-energy conformation of gp41.

What is the precise relationship between the above discussed class I viral fusion protein ectodomain structures and membrane fusion? Although this question is still open to discussion, recent studies support the idea that transitioning of the ectodomain from its native conformation into the trimer-of-hairpins conformation is directly linked to membrane fusion [108–110]. Along this line of reasoning, theoretical estimations showed that the packing of three C-terminal helices of gp41 into the hydrophobic grooves of the central triple-stranded coiled-coil can, by itself, produce sufficient free energy to overcome the activation barrier for lipid bilayer fusion [108]. However, several class I viral fusion proteins, including HA, possess a much shorter C-terminal helix than gp41 [96], suggesting that additional factors can also be important for coupling class I viral fusion protein refolding and membrane fusion.

Beta domains

In contrast to class I viral fusion proteins, class II viral fusion proteins are not predicted to contain helical coiled-coils, but are instead almost totally composed of antiparallel β sheet structures [93]. Other distinguishing characteristics of class II viral fusion proteins include a maturation step consisting in the cleavage of a second protein with which they heterodimerize, and the presence of an internally (not NH_2 terminally or NH_2 proximally) localized fusion peptide.

Recently, high-resolution native structures of two members of this group have been made available: that of the flavivirus tick-borne encephalitis protein E (TBE-E) (fig. 3C) [112] and alphavirus Semliki forest virus E1 protein [113]. Both proteins form elongated antiparallel homodimers that lie almost flat on the surface of the viral membrane. The internal fusion peptide is kept protected from interacting with the membrane at the dimer/heterodimer interface, being localized to the tip of the molecule opposite to that leading to the C-terminal transmembrane domain.

Activation of the fusogenic potential of E/E1 is triggered by the acid pH of the endosome. Activation involves dissociation of native dimers into monomers, exposure of the fusion peptide, and subsequent reorganization of the dissociated E/E1 monomers into trimers [114, 115]. Because the neutral pH E/E1 dimeric form is more labile than the low pH-treated E/E1 trimeric form, and because the dimer-to-trimer transition is an irreversible process, class II proteins are believed to exist in a metastable state in their native conformation, similar to the coiled-coil-based class I proteins [103, 113, 115, 116]. Moreover, a flexible hinge region located in the middle of the ectodomain of E/E1 has been postulated to serve as a pivotal point for bending the molecule in half upon low pH activation, so that the transmembrane domain and the fusion peptide can be brought into close proximity [113]. However, without detailed information of low pH-activated structures of E/E1, whether class I and class II viral fusion proteins share essentially the same fusion mechanism remains unclear.

Fusion peptides

Fusion peptides play a key role in successful membrane fusion, as demonstrated by the fact that most mutations within these sequences negatively affect viral/host cell membrane fusion and infection [73, 117–123]. Much insight into the mechanism of action of viral fusion peptides has been obtained by studying the interaction between synthetic fusion peptide analogues and liposomes [94]. The general ability of such synthetic peptides to induce liposome fusion suggests that viral fusion peptides are actively involved in the fusion process, rather than merely serving as membrane-anchoring devices.

An unusual property of viral fusion peptides is their high conformational flexibility in lipid membranes. An illustrative example is provided by the recently solved, high-resolution structures of the membrane-associated HA fusion peptide at neutral and acid pH [124]. At both pHs, the peptide adopts a kinked, predominantly helical amphipathic structure with an inverted 'V'-like shape, with the two 'arms' of the inverted 'V'-shaped structure penetrating obliquely into the cis monolayer of the bilayer. Amphipathic helical fusion peptides derived from HA [125, 126], HIV [127, 128], and SIV [129–131] have been shown to insert obliquely into lipid membranes, and such peptide orientation is apparently correlated with increased fusogenicity of intact viral proteins [132–134]. Returning to the fusion peptide structure of HA, the most important differences between the neutral and the low pH

form are found in the C-terminal portion of the peptide [124]. Here, an irregular segment present at neutral pH becomes helical at low pH, and two charged residues directed toward the interior of the membrane at neutral pH are relocated to the opposite side of the structure at low pH. Therefore, at the fusogenic pH, the surface of the peptide facing the membrane core becomes more hydrophobic, leading to a deeper penetration of the peptide into the membrane.

Why does fusion peptide penetration into the membrane favor membrane fusion? According to an increasingly recognized model originally proposed by Epand and coworkers [135, 136], fusion peptide insertion would perturb the lipid bilayer architecture of the membrane in such a way as to enhance its fusogenicity. This hypothesis is based on the capacity of a number of viral fusion peptides to destabilize lamellar lipid phases relative to nonlamellar inverted lipid phases, as well as in the good correlation observed between such activity and peptide-induced liposome fusion [130, 132, 135–142]. In the case of HA, the low pH-induced penetration of the inverted 'V'-shaped structure into the cis monolayer of the membrane may result in increased lateral pressure at the lipid hydrocarbon region, which, in turn, would impart negative curvature stress in the cis monolayer, facilitating formation of stalk-type nonlamellar lipidic structures and, thus, initiating membrane merger. Alternatively, or in addition, the bilayer-destabilizing function of the fusion peptide of HA may play an important role at later stages of the fusion process. This peptide has been shown to promote formation of inverted bicontinuous Q_{II} lipid phases, which possess structural elements resembling fusion pores (fig. 1B) [137]. Furthermore, Qiao et al. [73] demonstrated that replacement of certain amino acids within the HA fusion peptide led to cell-cell hemifusion, again suggesting involvement of this sequence in fusion pore formation. The fusion peptide of HA probably facilitates fusion pore opening by destabilizing the hemifusion zone through a curvature-independent mechanism [142], which may be related to its ability to decrease the bilayer rupture tension [55, 84, 143]. Of note, finally, is that several fusion peptides, including those of HIV-1 [140, 141, 144, 145] and class II viral fusion proteins [146] have been proposed to induce fusion through nonhelical, β-type structures.

Transmembrane domain

While the role of the ectodomain and its distinct regions in virus-mediated fusion has been investigated for a long time, the function of the transmembrane domain only came into focus more recently. A major breakthrough was made in 1993 with the demonstration that replacing the transmembrane domain of HA2 by a glycosylphosphatidylinositol anchor leads to membrane hemifusion, not complete fusion [68]. The same outcome (hemifusion) is obtained when the length of the HA2 transmembrane domain is reduced so that it becomes incapable of fully traversing the bilayer [75]. Similar results have also been obtained with other viruses [95]. Together, these findings suggest that completion of the fusion process via fusion pore formation requires the presence of a membrane-spanning transmembrane domain. As in the case of the fusion peptide, the transmembrane domain is thought to promote fusion pore formation by destabilizing the lipid bilayer structure of the hemifusion zone [69, 75, 78].

Mechanisms of fusion: coupling viral fusion protein refolding to lipid bilayer merging

The most extensively studied and best-characterized viral fusion protein is HA. Figure 4 displays three recent hypothetical models of HA-mediated membrane fusion. The essence in all three is the same: transitioning of HA2 from the native metastable conformation (top) into the more stable six-helix bundle conformation (bottom) is mechanistically and thermodynamically linked to the close apposition and merger of viral and cellular membranes.

According to currently favored models, the first step upon pH acidification is extension of the central coiled-coil of the ectodomain (green) (fig. 4A). This results in relocation of the N-terminal fusion peptide (bold arrow) to the tip of the structure, allowing for its insertion into the endosomal membrane. Then, the extended coiled-coil structure collapses through a helix-to-turn transition that reverses orientation of the C-terminal end (red). As a result, the fusion peptide and the transmembrane domain locally pull target and viral membranes toward each other, bringing them into close apposition. Completion of ectodomain reconfiguration into the triple-hairpin conformation, together with intrinsic bilayer-destabilizing activities of the fusion peptide and transmembrane domains, allows evolution through stalk/hemifusion intermediates and, finally, opening of a lipidic fusion pore.

Alternative models propose that the energy released through coiled-coil extension is used to form a high-energy defect at the viral membrane, which is critical to promote fusion of viral and endosomal membranes (fig. 4, B and C). Kozlov and Chernomordik [147] argued that the willingness of several HA2 molecules with their fusion peptides anchored into the viral membrane to achieve an extended coiled-coil conformation yields an upward pulling force that causes dimpling of the lipid bilayer of the viral envelope (fig. 4, B). Extreme curvature accumulated at the crest of the viral bilayer dimple (also known as the bilayer nipple [56]) permits a near-to-spontaneous advance from close bilayer apposition to fusion completion via the stalk mechanism. On the other hand, Bentz [148] postulated that extension of the central coiled-coil extracts

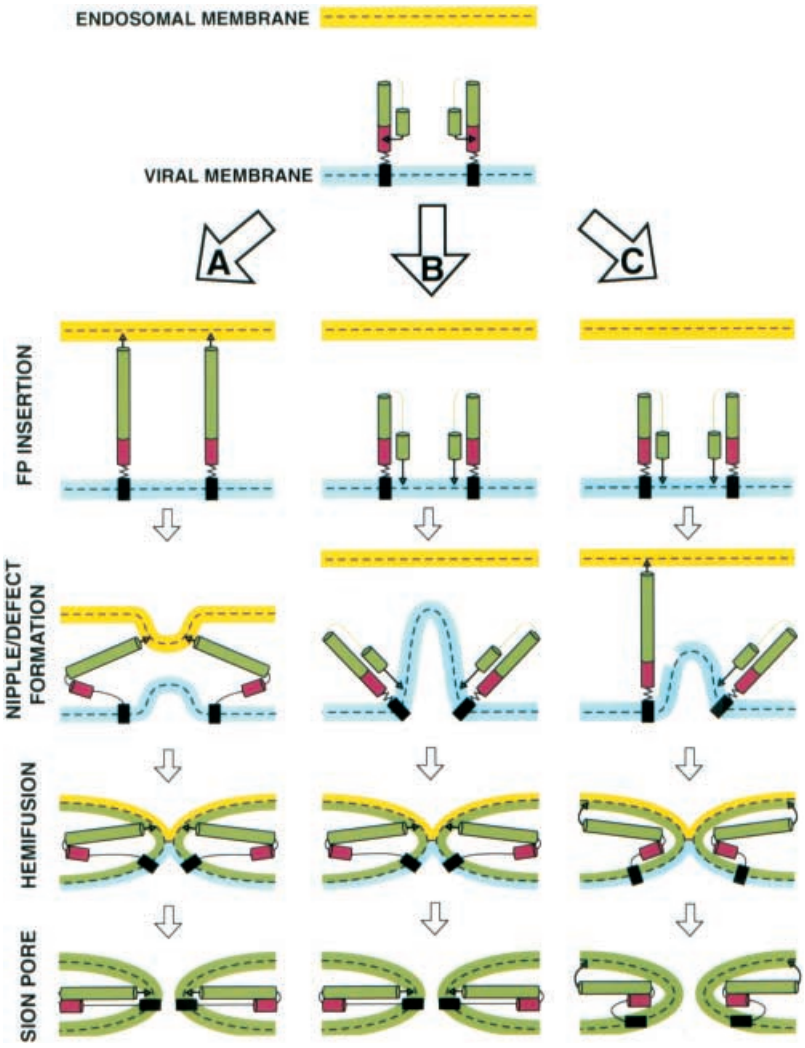

Figure 4. Mechanistic models coupling HA2 refolding and membrane fusion. Only HA2 monomers are shown for visual clarity. Top: native/metastable HA2 conformation, with N-terminal fusion peptides (FPs) (bold arrows) hidden within the protein structure. **A** Currently prevailing model. Upon low pH activation, native HA2 first shifts into a 'pre-hairpin' intermediate, in which the fusion peptides relocate to the top of the extended triple-stranded coiled-coil (green) for insertion into the endosomal membrane. Then, the ectodomain bends in half to pack the C-terminal helix (red cylinder) anti-parallel to the central coiled-coil (green cylinder). As the C-terminal transmembrane domain (black box) and the N-terminal FP (bold arrow) approach each other, they cause the drawing together and merger of target and host membranes via the stalk mechanism. **B** Upon activation, FP initially inserts into the viral membrane, and subsequent coiled-coil extension is coupled to the pulling-up of the viral membrane. Bending stress at the top of the dimple facilitates merger of interacting membranes via the stalk mechanism. **C** Here, extension of the coiled-coil results in extraction of the FP from the viral membrane, leaving a hydrophobic defect in that membrane. Lipids from the target membrane are then attracted to such a hydrophobic defect and fusion ensues via the stalk mechanism.

some of the fusion peptides from the viral membrane, creating a hydrophobic defect in that membrane which attracts lipids from the endosomal membrane (fig. 4C).

Finally, in addition to the features discussed in the above-presented models, clustering of multiple HA molecules at the fusion site is also generally recognized as an important contributor to the overall fusion process, both in mechanistic and energetic terms [72, 147–152].

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