

PROTEIN-LIPID INTERPLAY IN FUSION AND FISSION OF BIOLOGICAL MEMBRANES*

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■ **Abstract** Disparate biological processes involve fusion of two membranes into one and fission of one membrane into two. To formulate the possible job description for the proteins that mediate remodeling of biological membranes, we analyze the energy price of disruption and bending of membrane lipid bilayers at the different stages of bilayer fusion. The phenomenology and the pathways of the well-characterized reactions of biological remodeling, such as fusion mediated by influenza hemagglutinin, are compared with those studied for protein-free bilayers. We briefly consider some proteins involved in fusion and fission, and the dependence of remodeling on the lipid composition of the membranes. The specific hypothetical mechanisms by which the proteins can lower the energy price of the bilayer rearrangement are discussed in light of the experimental data and the requirements imposed by the elastic properties of the bilayer.

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1. INTRODUCTION

A complex choreography of membrane transformations, in which two membranes merge (fusion) and divide (fission), underlies the fundamental biological processes in normal and pathological conditions. Fusion and fission in exo- and endocytosis, in intracellular trafficking, in enveloped virus infection, and in many other reactions are all tightly controlled by protein machinery but also dependent on the lipid composition of the membranes. Whereas each protein has its own individual personality, membrane lipid bilayers have rather general properties manifested by their shapes, their elastic behavior in the course of deformations, and their resistance to structural changes.

Most of the current research on membrane remodeling is concentrated on the identification of proteins involved in fusion and fission and analysis of their conformational changes, which are supposed to induce membrane rearrangements. In this review we discuss membrane fusion and fission from a broader view. The starting point is a consideration of the physical factors that determine the tendency of the membrane bilayers to change their topology. Specifically, our analysis focuses on the elastic forces that drive membranes toward fusion or fission and on the intermediate membrane structures that constitute the pathways of these processes. The possible roles of proteins and lipids are addressed in the context of requirements imposed by these physical factors. In addition, a unifying consideration of fusion and fission helps us to identify the similar features of the two oppositely directed processes.

We discuss the emerging pathways of biological fusion and fission with special emphasis on the best-characterized examples of viral fusion reactions in which the proteins involved are reliably identified. Because a number of excellent recent reviews have summarized current understanding of the structure of identified and suspected fusion and budding-fission proteins, we only briefly touch upon some of these issues, those most relevant for our analysis. We also

discuss the dependence of membrane remodeling on the lipid composition of the membranes. Finally, we address the hypothetical mechanisms by which lipids and proteins can cooperate in lowering and paying the energy price of the required membrane transformation.

2. REMODELING OF LIPID BILAYERS: A JOB DESCRIPTION FOR PROTEINS

Membrane remodeling in biological systems necessarily includes the rearrangement of membrane lipid matrices. The lipid bilayer is stabilized against any structural changes by a powerful hydrophobic effect (1). Hence, remodeling requires energy, which either comes from the thermal fluctuations exerted by the membrane or is delivered to the membrane by specialized proteins.

The energy provided by thermal fluctuations within an experimentally reasonable timescale of 1 s has been estimated on the basis of electroporation studies (2) as $F_{\text{therm}} \approx 40k_B T$ (where $k_B T \approx 4 \cdot 10^{-21}$ J, or $\sim 10^{-21}$ cal, is the product of the Boltzmann constant, k_B , and the absolute temperature, T) (3).

If the energy required for membrane rearrangements exceeds F_{therm} , it has to be delivered by the proteins. In this section we formulate a possible job description for the proteins suggested by theoretical analysis of the energy consumed at each step of lipid bilayer remodeling. This discussion focuses mainly on the intermediate structures of the fusion reaction, which have been explored much more thoroughly than those of fission.

2.1 How Do Membranes Resist Remodeling?

The complicated membrane rearrangements consist of elementary constituents: ruptures and deformations. Two major theoretical approaches are currently used to describe membranes. The first is based on the modeling of membranes by powerful numerical methods (4, 5), and the second uses continuous description of membranes in terms of their average effective properties [reviewed in (6)].

The goal of the numerical approaches, such as molecular dynamics, is the simulation of the fusion reaction on the basis of the dynamics of each atom in the system (6). These methods are promising for the future, when our knowledge about the atom-atom interactions within the membrane and in the surrounding aqueous solution will reach a new level. Currently, these interactions are described by a number of fitting parameters, which cannot be measured directly.

A continuous approach, in contrast, does not account for the atomic details of the membranes. However, this method gives an effective description based on just a few measurable parameters and therefore provides a good background for qualitative and quantitative predictions. Thanks to these features, for the last three decades the continuous approach has been successful in understanding membrane behavior. Our analysis below is based on this approach.

2.1.1 RUPTURES Membrane rupture (Figure 1) exposes the hydrophobic interior of the bilayer to the aqueous solution. The energy of such exposure can be estimated from the hydrocarbon-water surface tension, $\gamma = 50$ mN/m (1). The energy of a circular monolayer rupture of radius r (Figure 1A) comes from the areas of the hydrophobic bottom and the hydrophobic wall,

$$F_{mr} = (\pi r^2 + 2\pi r d_m) \cdot \gamma,$$

where $d_m \approx 1.5$ nm is the monolayer thickness. For $r = d_m = 1.5$ nm, the energy of the rupture is $\sim 160k_B T$. A circular rupture of the whole bilayer (Figure 1B) has the energy $F_{br} = 4\pi r d_m \cdot \gamma$, which for the same radius $r = 1.5$ nm equals $\sim 350k_B T$. This hydrophobic energy is so large that lipid molecules at the edge of the pore reorient to cover the edge with the polar heads and to form the hydrophilic pore (Figure 1C).

2.1.2 DEFORMATIONS Among deformations, the most important for our purpose are bending of the membrane and tilt of the hydrocarbon chains (Figure 2A–C). It is convenient to characterize *bending of a monolayer* by the curvatures of a plane lying inside the monolayer close to the interface between the polar heads and the hydrocarbon chains of the lipids (Figure 2A, dashed lines) (7). *Bending of a bilayer* is described by the curvatures of the plane between the monolayers (Figure 2B, dashed lines). Although a surface bending is characterized by two principal curvatures (Figure 2D), in practice one uses their combinations, called the total curvature, $J = c_1 + c_2$, and the Gaussian curvature, $K = c_1 \cdot c_2$, which have a profound geometrical meaning. For mathematical reasons, the Gaussian curvature is only rarely relevant for the description of membrane deformations. For a monolayer, the curvature is defined as positive, $J > 0$, if the surface bends toward the polar heads (Figure 2A). For the bilayer of a closed vesicle or a cell, positive curvature corresponds to bending toward the outside medium (Figure 2B).

If there are no forces acting on its surface, a lipid monolayer adopts a curvature J_s called the spontaneous curvature (8, 9). Lipid molecules are then characterized by their effective shapes (10). The spontaneous curvatures of the monolayers of the most important lipids have been measured by Rand and collaborators (7, 11–13). The one-chain oleoyl lysophosphatidylcholine (LPC) has the effective shape of an inverted cone described by a positive spontaneous curvature of $1/(5.8$ nm) (12). Regular two-chain lipids are almost cylindrical and have a slightly negative spontaneous curvature such as $-1/(87.3$ nm) for dioleoylphosphatidylcholine (DOPC). Finally, the two-chain lipids having relatively small polar heads have strongly negative spontaneous curvatures such as $-1/(2.8$ nm) for dioleoylphosphatidylethanolamine (DOPE) and even $-1/(1.01$ nm) for dioleoylglycerol (13). Lipids of pronounced positive or negative spontaneous curvature are often referred to as nonbilayer lipids.

The spontaneous curvature of bilayers, J_s^B , is generated by the difference between their monolayers. According to the bilayer-couple hypothesis (14), the bilayer spontaneous bending is produced by a difference in the areas of the outer, A_{out} , and inner, A_{in} , lipid monolayers, resulting in J_s^B proportional to $A_{\text{out}} - A_{\text{in}}$.

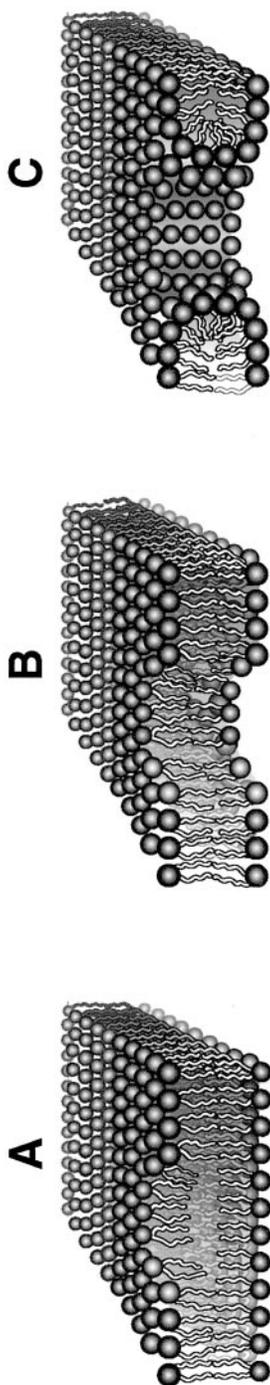


Figure 1 Bilayer rupture. (A) Hydrophobic defect in one monolayer. (B) Hydrophobic pore. (C) Hydrophilic pore.

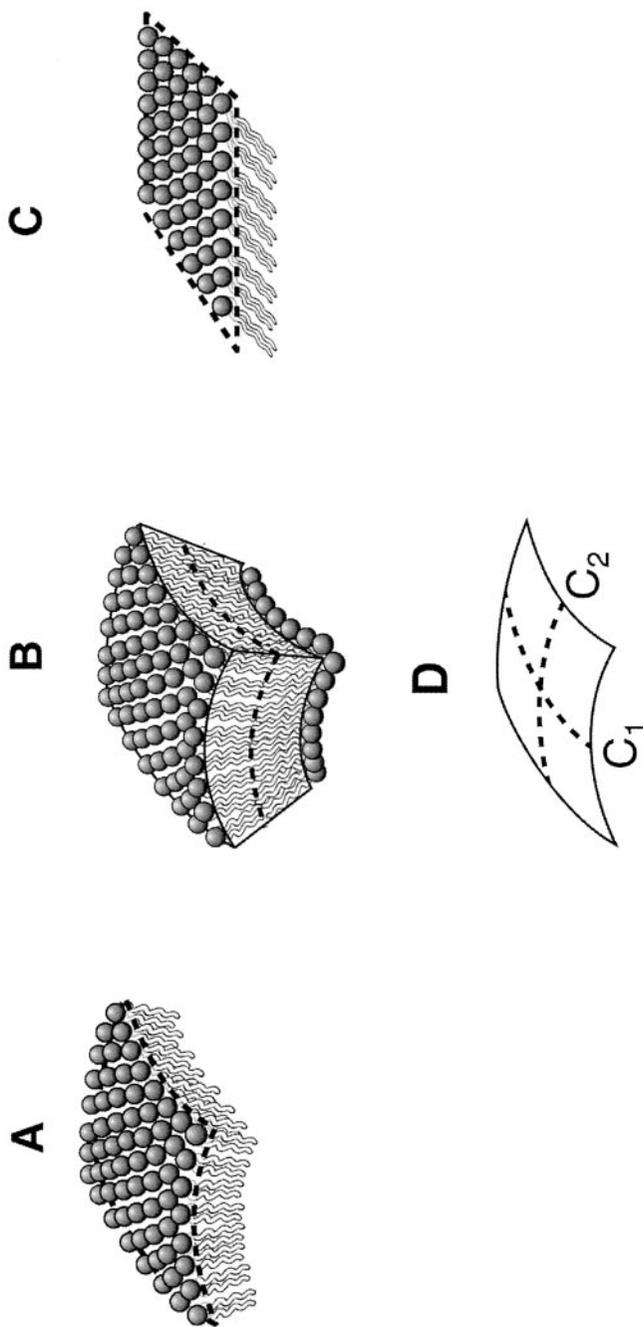


Figure 2 Membrane deformation. (A) Bending of lipid monolayer. (B) Bending of lipid bilayer. (C) Tilt of hydrocarbon chains. (D) Curvature of the surface, which represents the membrane.

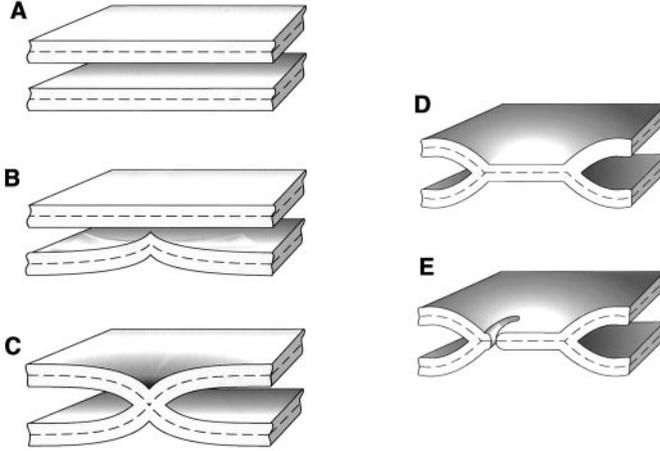


Figure 3 Fusion of lipid bilayers. (A) Establishment of membrane contact. (B) Pointlike protrusion at the prefusion stage. (C) Fusion stalk. (D) Hemifusion diaphragm. (E) Cracklike fusion pore.

Another reason is a difference in the spontaneous curvatures of the outer, J_s^{out} , and inner, J_s^{in} , monolayers, leading to J_s^B proportional to $J_s^{\text{out}} - J_s^{\text{in}}$.

Under external forces, a membrane bends with respect to its spontaneous curvature. The bending energy F_{bend} as a function of deformation is given by: $F_{\text{bend}} = (1/2)A \cdot \kappa \cdot (J - J_s)^2$, where A is the membrane area and κ is the bending modulus or bending rigidity (8). The rigidity of a lipid monolayer is $\kappa_m \approx 10k_B T$. For a bilayer it is twice as large, $\kappa_b \approx 20k_B T$. Using the concept of bending energy, one can calculate the elastic energy of a spherical vesicle of radius R whose membrane has zero spontaneous curvature, $J_s = 0$, area $A = 4\pi R^2$, and total curvature $J = 2/R$. This energy does not depend on the vesicle radius and is $F_{\text{bend}} = 8\pi\kappa_b \approx 500k_B T$.

One can also estimate the energy of the hydrophilic pore (Figure 1C) as the bending energy of the monolayer covering the pore edge. We obtain the energy per unit length of the edge as $f_{\text{pore}} = (1/2)\kappa_m(1/d_m) \approx 3k_B T/\text{nm} \approx 10^{-11}$ J/m. This energy, also referred to as the line tension, is close to those measured experimentally (15) and estimated in a more sophisticated way (16). Based on this value the edge energy of a pore of 1-nm radius can be estimated as $\sim 20k_B T$.

Treatment of the tilt of hydrocarbon chains (Figure 2C) requires a more complicated modeling and is presented in detail elsewhere [(17) and references therein].

2.2 Job 1: Bringing the Membranes Together

Before remodeling starts, the membranes have to establish a contact (Figure 3A). The bilayers, which do not carry an electric charge, tend to approach each other

spontaneously up to the equilibrium distance of 2–3 nm (18). The initial distances between biological membranes are much larger, 10–20 nm, because of the electrostatic repulsion between the bilayers and the steric interaction of membrane proteins. Thus, fusion/fission proteins have to bring membrane lipid bilayers into reasonably close contact (a distance of a few nanometers), allowing the downstream fusion/fission stages to proceed. This aim might require the proteins to either pull or push the membranes toward each other and to bend them locally to minimize the area of the strongest intermembrane repulsion (19).

2.3 Job 2: Prefusion/Prefission Stage

Merger of the contacting membrane surfaces requires the formation of some transient membrane discontinuities. The energy price of this stage includes the local membrane approach to almost zero distance against strong intermembrane repulsion, the energy of rupture of the merging monolayers, and the deformation energy of the monolayers accompanying their local approach and rupture.

Earlier attempts to analyze this stage of fusion (19) predicted large contributions to the energy from intermembrane repulsion and hydrophobic monolayer discontinuities. This result was related to the assumption that membranes approach each other at rather large areas on the tops of smooth membrane bulges where strong hydration repulsion (18) prevents them from establishing a dehydrated contact. We suggest a different configuration of the prefusion intermediate, which is illustrated in Figure 3B. Such pointlike protrusions must exhibit a minimal repulsion from the apposing membrane, thus producing a minimal contribution to the energy. Formation of a pointlike dehydrated contact between the membranes will decrease the hydrophobic energy of the monolayer rupture. Pointlike protrusions have not been considered before because of the seemingly high energy of sharp bending at the top of the protrusion. However, recent developments of the elastic model of membrane rearrangements, including tilt deformation (17), have demonstrated the feasibility of structures of this kind. A similar sharp ridgelike fluctuation of the membrane neck might be considered as a possible prefission intermediate. Theoretical analysis of these prefusion and prefission intermediates is a matter for future work.

Proteins can facilitate this stage by inducing local dehydration of the membrane contact and elastic stresses supporting membrane fluctuations.

2.4 Job 3: Fusion Stalk and Hemifission Intermediate

The junction of the transient discontinuities of the contacting monolayers of two membranes yields a first intermembrane lipid connection (Figure 3C) called the fusion stalk (20). In the case of fission, this stage corresponds to fission of only the internal monolayer of the membrane neck.

The evolution of ideas on the fusion stalk, beginning from the original model (21), ran into an important roadblock in 1993 when Siegel formulated the energy crisis of the stalk model. When the energy of the hydrophobic interstice, the

structural defect unavoidably emerging in the stalk middle, was considered, the energy of a stalk was found to be prohibitively high (22).

This energy crisis was solved by optimized packing of the hydrocarbon chains within interstices for the case of strongly bent bilayers in a very close (~ 1 nm) contact (23) and then for the canonical case of flat membranes at arbitrary intermembrane distances (17), along with finding the stalk shape that minimizes its bending energy (17, 24). The stalk structure (Figure 3C) suggested in (17) combines deformation of bending, tilt, and splay of the monolayers with optimized monolayer shape and no vacuum voids inside the interstices. This model predicts feasible energies for stalk without any requirements on prefusion membrane deformation and at all experimentally relevant intermembrane distances. For the membranes of DOPC the predicted stalk energy is $\sim 45k_B T$ (17), which exceeds by $\sim 5k_B T$ the characteristic thermal energy $F_{\text{therm}} \approx 40k_B T$ (see above). The more negative the spontaneous curvature of the contacting monolayers is, the lower the energy of the stalk becomes. For DOPE, which is known to undergo spontaneous fusion in lipid mesophases, the stalk energy is negative, $\sim -30k_B T$, meaning that the stalk is energetically favorable for this lipid and its formation does not limit the fusion process. Stalklike structures have recently been detected by X-ray diffraction for a dehydrated lipid of the negative spontaneous curvature (25).

The task of the fusion proteins might be to deliver the energy required for stalk formation in a bilayer of biological lipid composition. Protein might generate bilayer stresses that relax during stalk formation and release the energy needed.

Analysis of the hemifission intermediate is currently being undertaken in the spirit of the updated stalk model (Y. Kozlovsky and M.M. Kozlov, in preparation).

2.5 Job 4: Hemifusion Diaphragm

Three different scenarios have been suggested for evolution of the stalk into a fusion pore, which connects the aqueous volumes bounded by the fusing membranes. In the stalk-pore hypothesis (15, 26), radial expansion of the stalk results in a dimpling of the distal monolayers toward each other and the formation of a single bilayer (21, 27), referred to as a hemifusion diaphragm (HD) (Figure 3D). Formation of a pore in the HD (26) completes fusion. To allow formation of the pore edge, the HD radius has to reach at least a few nanometers. An alternative model proposes that the fusion pore forms directly from the stalk, avoiding the stage of stalk expansion (22, 23). The third approach, based on numerical simulations of bilayer fusion, suggests that the initial fusion stalk extends anisotropically into an elongated stalk, which induces the formation of holes in the stalk vicinity in both fusing membranes (5). Fusion of the rims of the two apposing holes yields a fusion pore.

Very recently, stalk evolution has been analyzed using the elastic model of tilt and bending deformations (28). We have found that a circular HD is always more favorable energetically than an elongated stalk. The intrinsic tendency of a fusion

stalk to expand into an HD is controlled by the spontaneous curvature, J_s , of the contacting monolayers of the fusing membranes. For positive or moderately negative J_s , such as that of DOPC, the HD does not form spontaneously. However, even in these conditions an HD can form provided that its rim is subject to an external force pulling the HD apart. An obvious job for proteins is to induce this pulling force.

2.6 Job 5: Fusion Pore Formation

Formation of the edge of the hydrophilic pore is facilitated by the positive spontaneous curvature of the HD monolayers (15). Opening of a fusion pore is also promoted by the lateral tension, σ , developed in the diaphragm. The characteristic time of pore formation depends on both the lateral tension and the area of the stressed membrane. The larger the area, the higher the probability of pore formation within a given time span and under a given tension (15). The usual tension leading to pore formation within seconds in the membranes of unilamellar vesicles of a diameter of ~ 40 nm is $\sigma \approx 10$ mN/m (29). What mechanism can produce this tension in an HD?

The recent HD model predicts a very large tension, $\sigma \approx 20$ mN/m, generated by the elastic stresses of tilt and bending in the narrow region along the diaphragm rim (28), which may lead to formation of a cracklike fusion pore expanding along the diaphragm rim (Figure 3E). This is different from the usual circular pores formed in homogeneously stressed membranes.

A task for proteins is to pull the HD rim apart, thus increasing the probability of pore nucleation. The analogues of the HD and fusion pore in the case of fission are not obvious at this stage and are a matter for future work.

The importance of the bending deformations in membrane remodeling, and in particular the role of stalk and pore intermediates in fusion, have been verified by the experimental studies on protein-free bilayers.

2.7 Lipid Bilayer Fusion Observed Experimentally

Bringing two protein-free bilayers into close contact is not sufficient for fusion. Bilayers formed from phosphatidylcholine (PC), a major constituent of the membranes of mammalian cells, do not fuse when kept for hours and even days in very close contact (~ 3 nm). Only application of tension or dehydration of the contact zones with high concentrations of polyethylene glycol fuses bilayers of this composition (15, 30, 31). However, under certain conditions bilayers do fuse [reviewed in (15, 31)]. In addition, reliable evidence is provided for the existence of the hemifusion state. For instance, for two fusing planar bilayers, the specific electrical capacitance, and thus the thickness of the contact zone, coincides with that of a single bilayer (15). In liposome-planar bilayer and liposome-liposome fusion, mixing of the lipids of the contacting monolayers of the membranes proceeds when neither lipid mixing between distal monolayers nor a fusion pore is detected (32–35).

The hemifusion phenotype can represent the end-state of the fusion reaction (15, 33, 36, 37). Alternatively, the reaction proceeds to the opening of a fusion pore (32, 33). Hemifusion depends on the lipid composition of the contacting monolayers of the membranes (15, 31, 35, 36, 38). Lipids of positive and negative spontaneous curvature (e.g., LPC versus phosphatidylethanolamine, PE, or oleic acid, OA), respectively, inhibit and promote hemifusion. In contrast to hemifusion, the subsequent opening of a fusion pore depends on the lipid composition of the distal membrane monolayers. Lipids that promote hemifusion (OA and PE) inhibit pore formation, and vice versa: LPC, which inhibits hemifusion, promotes pore formation (15, 36). Thus, fusion dependence on lipids in the contacting membrane monolayers supports the hypothesis that fusion proceeds through stalk intermediates. The effects of the lipids in the distal monolayers are consistent with pore formation in HDs as predicted by the stalk-pore model [see above and (39)].

The dependence of bilayer fusion on the lipid composition has also been discussed in terms of the correlation between fusion promotion and increase in the hydrophobicity of the bilayer surface, increase in bilayer tension, and the presence of lipid packing defects, including formation of so-called extended conformations of the lipids (40–45).

2.8 Lipid Bilayer Fission Observed Experimentally

Even a narrow lipid bilayer tube resists fission. Lipid tethers of a few nanometers in diameter can be very stable (46). However, under certain conditions bilayers do divide. One of the experimental models for studying fission of protein-free bilayers is based on a membrane tube formed by fusion of two black lipid membranes (15). Fission of such tubes is driven by surface tension. Shape transformation and fission can also be driven by the domain boundary in heterogeneous membranes (47), as has been observed for vesicles composed of dimyristoyl phosphatidylcholine and cholesterol and for sphingomyelin vesicles at the temperature of the lipid phase transition that generates inhomogeneities (48). Finally, enzymatic formation of ceramide-enriched microdomains in fluid giant vesicles composed of phosphatidylcholine and sphingomyelin causes budding and release of the smaller vesicles (49). In this case, vesiculation is driven by the spontaneous curvature and area difference between the monolayers generated by the asymmetric production of the ceramide. The underlying effects of the spontaneous curvature of the bilayer on the shapes of the protein-free vesicles are discussed in (50).

3. REMODELING OF BIOLOGICAL MEMBRANES

The intermediate structures in the fusion and fission pathways for biological membranes are better characterized for membrane fusion and, in particular, for fusion mediated by viral fusion proteins (51, 52). In this section we compare the

intermediates of the prototype biological fusion reaction mediated by influenza hemagglutinin (HA) with those identified for protein-free lipid bilayers. We then briefly discuss the much less characterized membrane pathway of protein-mediated fission.

3.1 Fusion: Experimental Approaches

Numerous enveloped viruses enter the host cells either by endocytosis or by direct fusion of the viral envelope to a plasma membrane (53). In the former mechanism, fusion proteins such as HA of influenza virus A are activated by low pH within the endosome. In the latter mechanism, fusion proteins such as HIV gp120/41 are activated at neutral pH by complex interactions with cellular receptors.

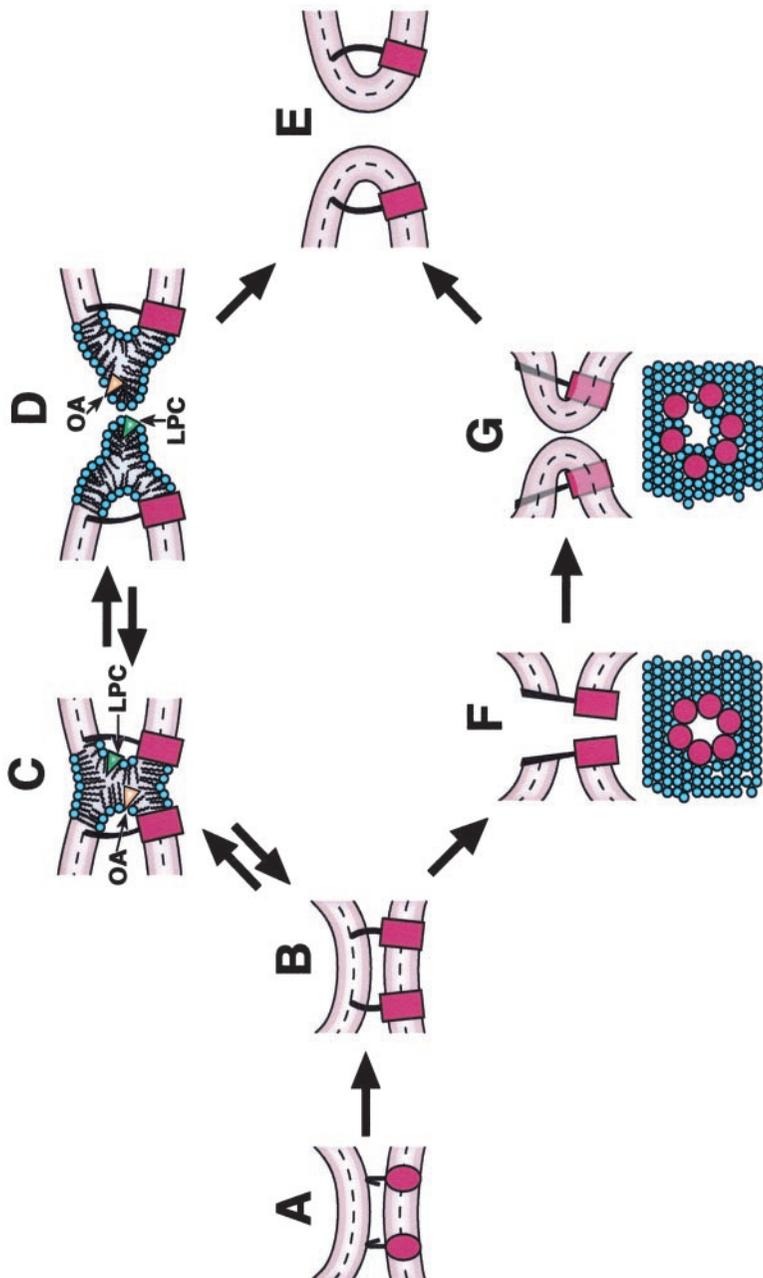
Fusion is usually studied in experimental systems, which are much simpler than actual virus entry into a host cell. These *in vitro* systems include low-pH-triggered or receptor-triggered fusion of virus to liposomes or target cells. Alternatively, viral fusion proteins are expressed in cells. Fusion of these cells with target cells or with lipid bilayers is assayed as redistribution of membrane probes and aqueous content probes using fluorescence microscopy, spectrofluorimetry, and electrophysiology.

3.2 Fusion Phenotypes and Hypothetical Pathways

Under optimal conditions, fusion is usually too fast to be dissected into distinct stages. Thus, current understanding of the pathway of viral fusion (Figure 4A–E) is based mostly on experiments in which fusion was slowed down by lowering temperature, modifying fusogenic proteins, decreasing their numbers, and/or altering lipid composition to that unsuitable for fusion. Blocking, or at least slowing down, of fusion progression beyond a certain stage allows characterization of that stage.

Some of the phenotypes of biological fusion resemble those identified for protein-free lipid bilayers. Early fusion pores (Figure 4D) in HA-mediated fusion

Figure 4 Two hypothetical pathways in protein-mediated membrane fusion. (A) Membrane expressing fusion protein (for instance, HA) in contact with the target membrane. (B) Conformational change in the activated fusion proteins. (C) Fusion stalk. (D) Opening of a lipidic fusion pore. (E) Fusion pore expansion. (F, G) Alternative pathway involves formation of a proteinaceous pore (F), which then expands to allow membranes to establish lipidic connection (G). Stalk formation (C) is facilitated by cone-shaped lipids (e.g., OA), and hindered by inverted-cone-shaped lipids (e.g., LPC). Lipid dependence of the pore (D) is opposite to that in the stalk. Dashed lines show the boundaries of the hydrophobic surfaces of monolayers. The diagrams of intermediates F and G include a view from below with large and small circles to represent proteins and lipid polar heads, respectively.



(54, 55) open abruptly, fluctuate, and then either close or expand irreversibly with characteristic electrophysiological features similar to those in bilayer fusion (33). As in fusion of bilayers, protein-mediated fusion yields a hemifusion phenotype (56) (Figure 4C). The unambiguous evidence for HA-mediated hemifusion, lipid mixing in the absence of a fusion pore, has come from combining the most sensitive electrophysiological assay for fusion pores with fluorescence microscopy used to assay lipid probe redistribution (57, 58). Additional fusion intermediates, characteristic only of protein-mediated fusion, have also been identified. In contrast to lipid bilayer fusion, the opening of a small fusion pore in HA-mediated fusion precedes the onset of detectable lipid mixing (54, 59). Lipid flow between membranes is also restricted in another type of HA-formed fusion intermediate, which can be transformed into complete fusion (lipid- and content-mixing) only with treatments known to destabilize the HD (58, 60). This fusion phenotype apparently represents local hemifusion (58, 61, 62) and is referred to as restricted hemifusion because lipid flow through these structures is restricted by activated HAs. Formation of the restricted and reversible (61) hemifusion connections happens as fast as the opening of a fusion pore but requires less HA. Therefore, the opening of a pore is likely preceded by formation of multiple restricted hemifusion sites (61, 63).

Even if the fusion pathway under biologically relevant conditions involves membrane structures, which are arrested and identified as distinct phenotypes, the specific fusion pathway is always a matter of conjecture rather than a direct experimental finding. For instance, it remains to be verified that pores are formed in the hemifusion structures and that large fusion pores are formed by expansion of initial small pores. However, the set of available pieces of the puzzle is consistent with the hypothesis that fusion starts with local hemifusion, which then breaks into a small pore that subsequently expands (Figure 4A–E). Note that the prediction of a cracklike pore propagating along the HD rim (Section 2.6) (28) is supported by the observation in HA-mediated fusion of the lack of movement of aqueous dyes while total fusion pore conductance increases (54). Elastic stresses generated along the perimeter of HD might also explain the intriguing recent finding that homotypic fusion of yeast vacuoles proceeds along the rim of the membrane contact area (63a).

3.3 Budding-Fission: Intermediate Stages

In contrast to fusion, the pathway of budding-fission reactions has been well studied in only one *in vitro* system, receptor-mediated endocytosis of transferrin (Tfn) in semi-intact or perforated cells (64–66). Narrowing of the cross section of the neck was assayed as changes in the accessibility of Tfn in the budding membrane compartments for different probes. The size of the probes varied from many nanometers in the case of antibodies and avidin to a few angstroms in the case of 2-mercaptoethanesulfonic acid. Three sequential stages of budding fission have been identified: (a) the budding initiation, where receptor-bound Tfn is accessible for all probes; (b) the constricted bud, where Tfn is inaccessible to the bulky probes while still accessible to the small probe, 2-mercaptoethanesul-

fonic acid; and finally, (c) the sealed vesicle, where Tfn is inaccessible for all probes. Note that at the sealed vesicle stage, this experimental approach does not distinguish between a bud that has undergone hemifission but is still connected to the initial membrane and a completely separated vesicle.

4. PROTEINS THAT DRIVE MEMBRANE REMODELING

4.1 Fusion Proteins

Do different fusion proteins share any structural motifs reflecting their common functionality? A definitive answer to this question is hindered by the paucity of available information about these proteins along with the fact that most (if not all) reliably identified fusion proteins are viral envelope proteins. The structures and properties of the best-characterized viral fusion proteins have recently been reviewed in depth [for instance, HA (67), gp120/gp41 (67–69), E protein of flaviviruses (70), and G protein of rhabdoviruses (71)]. Below, we briefly discuss only those properties of the fusion proteins that appear to be most relevant for lipid bilayer remodeling.

4.1.1 OVERALL STRUCTURE OF THE FUSION PROTEIN AND ITS REFOLDING UPON ACTIVATION All characterized viral fusion proteins are anchored in the envelope by transmembrane domains (TM). Although for some proteins there is significant latitude in the TM sequence that supports fusion (72, 73), the specific anchor by which protein is attached to a membrane is of importance for its fusogenic activity. Replacing the TM of HIV gp41 (74, 75), HA (56, 57), and parainfluenza virus fusion protein (76) with a lipid anchor blocks the ability of these proteins to form expanding fusion pores. Modifying the TM either of HA (73, 77, 78) or of the fusion protein of vesicular stomatitis virus (79, 80) also inhibits fusion.

On the basis of the overall structure of the ectodomain, the majority of the viral fusion proteins can be divided into two classes (81). In class 1 (for instance, HA and gp120/gp41), the fusion peptide (see below) is located at or near the NH₂ terminus of the fusion protein created by the proteolytic cleavage upon protein maturation. Activated proteins of this class share the common 6-helix bundle, a hairpin arrangement with a central α -helical coiled-coil domain. In this very stable structure, the fusion peptide and TM are located at the same end of the rodlike molecule.

Class 2 proteins (for instance, E protein of flaviviruses and E1 protein of alphaviruses such as Semliki Forest virus) have the fusion peptide in an “internal” location rather than at the NH₂ terminus of the protein. In this case, proteolytic cleavage leading to mature conformation occurs in the accessory protein rather than in the fusion protein itself. Class 2 proteins are not predicted to form coiled-coils and contain predominantly β -strand secondary structures.

As a rule, conformational change of the activated proteins of both classes is profound and likely releases significant conformational energy. For most viral fusion proteins, with the intriguing exception of G protein of rhabdoviruses (71), activation in the absence of a target membrane results in the irreversible functional inactivation of the proteins. Although fusion can, in principle, be mediated by some transient protein conformations that are closer to the initial than to the stable inactivated form of the protein (82–84), at least some features of the major restructuring are important for fusion. In particular, HA mutations expected to block the extension of the central coiled-coil (85) inhibit fusion.

4.1.2 FUSION PEPTIDE The early work on the HA-mediated fusion identified the functional importance of the fusion peptide, i.e., the NH₂ terminus of the HA2 subunit of HA (67) that inserts into the membranes upon activation (86). Mutations in this region of ~20 amino acid residues inhibit fusion (87, 88). NH₂-terminal or internal peptides similar to the fusion peptide of HA have been found in many other viruses.

The structures of the different fusion peptides and the effects of these peptides on lipid bilayers have been examined extensively (88–92). These studies indicate that the functional role of the membrane insertion of the fusion peptide is not limited to just establishing an additional membrane anchor. It is also clear that the functionality of the fusion peptide in the context of the entire protein cannot be fully explained by studying the synthetic peptide/bilayer interactions. For instance, changes in the HA fusion peptide, which at the level of corresponding synthetic peptides significantly alter peptide ability to modulate the spontaneous curvature of lipid monolayers, do not affect the fusogenic properties of the whole protein (92).

The existence of a distinct fusion peptide—usually a stretch of 10–30 amino acid residues that is amphiphilic, highly conserved, critical for fusion, hidden in the initial conformation of the protein, exposed in the activated conformation and then inserted in the membrane—is widely assumed to be one of the most important prerequisites for the generic fusion protein. However, the influx of additional structural information and the broadening of the collection of characterized fusion proteins somewhat blur the exact meaning of the term fusion peptide. A conserved uncharged sequence in the fusion proteins of rhabdoviruses, recognized as a fusion domain and found to insert into the target membranes, contains some polar amino acids and is much less hydrophobic than other fusion peptides (71). Ectodomains of several fusion proteins have multiple regions with the properties expected for fusion peptides (93–95).

It remains possible that the functional role of some of the protein regions identified as fusion peptides is not only limited to direct interaction with the membranes but also, or even instead, involves supporting or destabilizing certain protein structures. In particular, fusion peptides can mediate some functionally important interactions between adjacent proteins (96), or within the same protein. For instance, interactions between the fusion peptide and the TM of a fusion

protein might be instrumental in transition from hemifusion to an opening of an expanding fusion pore (87, 88, 97, 98).

4.1.3 POSTACTIVATION OLIGOMERIZATION Activated fusion proteins form high-order oligomers. HA aggregation is detected as a loss of HA mobility along the membrane surface (99). Morphologically, HA aggregation is seen as the transformation of the well-defined spikes of the neutral-pH form of HA into a “thick layer of entangled thin threads” (100). Low-pH-induced aggregation at the surface of the membrane has also been reported for the large fragment of the HA2 subunit, FHA2 (101). Two specific HA domains exposed in activated HA, the fusion peptides (102, 103) and the kinked regions of HA2 (residues 106–112) responsible for the aggregation of FHA2 (101, 104), have been implicated in interactions of HA trimers.

Fusion proteins other than HA also assemble into high-order oligomers. Multimeric aggregates are reported for HIV gp41 (105) and dengue virus E protein (106) released from the interactions stabilizing the initial form of fusion proteins. Under conditions of fusion, baculovirus gp64 forms aggregates containing up to 10 trimers (107). Stability of the gp64 trimers is a prerequisite for formation of these aggregates, indicating that trimers serve as a fundamental building block. Destabilization of the gp64 trimers by reducing the intermonomer S–S bonds completely abolishes both aggregation and fusogenic activity of this protein (107).

Not only does activation promote interactions between adjacent fusion proteins, but vice versa: Interactions between adjacent fusion proteins promote activation. For the same conditions of activation (pH and temperature), the percentage of low-pH-activated HA increases with higher surface density of cleaved HA trimers capable of undergoing such conformational changes (96). There is an early reversible form of low-pH-activated HA from which HA can still revert to the initial conformation (108). Intertrimer interaction shifts HA restructuring toward irreversible stages. Reversible stages prior to a major restructuring have been discussed for fusion proteins of some other viruses [e.g., flavivirus (109), rabies virus (71), and HIV (110, 111)] and might be a common feature of different fusion proteins.

Interactions between fusion proteins, a distinct reversible stage of refolding, and positive cooperativity of the activation might be crucial for the synchronized discharge of the conformational energy of the multiple adjacent proteins in a hypothetical fusion machine. The notion that fusion is mediated by a concerted action of multiple fusion proteins is supported by numerous functional studies (58, 61, 99, 107, 112–117).

4.2 Budding-Fission Proteins

4.2.1 INTRACELLULAR BUDDING The protein machinery involved in budding and fission has been reviewed recently (66, 118, 119). In brief, bending of the membranes into spherical buds in different pathways of intracellular trafficking and virus

budding-off is driven by the layer of proteins (coat) assembled at the membrane surface. Intracellular trafficking utilizes clathrin, COPI, and COPII coats.

In addition, trafficking requires some cylinder-forming proteins, which constrict membranes into tubular shapes and are apparently involved in the formation and constriction of membrane necks connecting the buds to the nearly flat initial membrane.

The first discovered cylinder-forming protein, dynamin, a 100-kDa GTPase, self-assembles into helices on the membrane surface and constricts flat membranes into collared tubes with an external diameter of ~ 50 nm (120–122). Dynamin can bend membranes in concert with clathrin and on its own (120, 121, 123). The budding pathway driven by dynamin without involvement of sphere-forming coats is referred to as fission-only and is implicated in a kiss-and-run mechanism of exocytosis (124).

Recently, additional proteins, amphiphysin 1 and 2 (121) and endophilin (125), have been found to tubulate the membranes both on their own and in concert with dynamin. Tubes formed by either amphiphysin or endophilin have diameters similar to those formed by dynamin. Another protein, epsin, binds to phosphatidylinositol-4,5-bisphosphate-containing liposomes and bends them into tubes with the outer diameter of approximately one third of that of the tubes formed by the dynamin (125a). Epsin-induced membrane invagination may go hand in hand with clathrin polymerization and AP2 complex recruitment.

4.2.2 INTRACELLULAR FISSION Most sphere-forming protein coats are capable of severing the membrane necks and separating the coated vesicles from the initial membranes (126, 127). The exception is the clathrin coat, which does not pinch off the coated buds without the assistance of its cylinder-forming partners (128). Fission of membrane tubes mediated by dynamin correlates with its GTPase activity (120, 121). Amphiphysin supports dynamin-induced tube fragmentation, and endophilin inhibits it (125).

Although dynamin can induce fission on its own, in biologically relevant situations its function in fission can include, or even be limited to, recruitment of additional proteins. A proposed candidate is endophilin, the protein involved in synaptic vesicle formation, which, in addition to its cylinder-forming properties, exhibits acyltransferase activity (129, 130). Strikingly, another acyltransferase, CtBP/BARS, is reported to induce constriction and fission of Golgi tubes (131).

Tightly controlled reactions of intracellular budding fission involve many other important proteins, including phosphatidylinositol (PI)-transfer proteins (132) and protein kinase D (133). The yet unresolved challenge is to reliably distinguish proteins that control the reactions from those that actually mediate them.

4.2.3 VIRAL BUDDING AND FISSION Budding of the enveloped viruses releases them into the extracellular space (118). For influenza virus, cell expression of just one viral matrix protein, M1, which assembles under the membrane, is sufficient to bend cell membrane into buds and release spherical viruslike particles (134).

Similarly, just two flavivirus proteins, envelope Glycoproteins E and prM, assemble at the membrane and drive the formation and release of small subviral particles (135).

4.3 Lipids in Biological Fusion and Fission

4.3.1 FUSION AND NONBILAYER LIPIDS Protein-mediated fusion is sensitive to membrane lipid composition. Some lipids are of importance for specific fusion reactions. For instance, PE is important for fusion of Golgi membranes (136). Sphingolipid and cholesterol are strictly required for fusion mediated by Semliki Forest virus (137, 138). Polyphosphoinositides play a signaling role in membrane trafficking (139).

Although lipids can undoubtedly control the local concentration and activity of specific proteins, some lipid effects on fusion apparently reflect changes in the lipid bilayer propensity to fuse. The latter effects are strikingly universal and follow the predictions of the stalk-pore hypothesis. For instance, LPC, a lipid of positive spontaneous curvature, which inhibits fusion of protein-free bilayers, also inhibits disparate biological fusion reactions if added to the contacting membrane monolayers (Figure 4C). This lipid reversibly blocks fusion mediated by fusion proteins of baculovirus (140), Sendai virus (141), rabies virus (142), HIV (98), paramyxovirus SV5 (143), and Semliki Forest and avian leukosis viruses (personal communication with J. Wilschut and with G. Melikyan). LPC also inhibits a number of nonviral fusion reactions triggered by Ca^{2+} , GTP- γ -S, and GTP [reviewed in (144)].

Although inhibition of biological fusion by these amphiphiles is consistent with their expected effects on the energy of the stalk and HD, alternative interpretations have been also discussed (145). The most important alternative suggested is that LPC affects fusion by direct interaction with the exposed amphiphilic regions of the fusion protein (140, 146). However, a recent report showing very weak interaction between LPC and activated HA (147), along with an earlier analysis (145), argues against this interpretation.

In contrast to lipids of positive spontaneous curvature, lipids of negative curvature such as PE, diacylglycerol (DAG), and *cis*-unsaturated fatty acids (e.g., OA) promote biological fusion (142, 144, 148) (Figure 4C).

No specific lipid moiety is responsible for this dependence of fusion on nonbilayer lipids. Different naturally occurring nonbilayer lipids and synthetic surfactants that are quite dissimilar in the structures of their hydrocarbon chains and polar heads reversibly inhibit or promote fusion in specific correlation with their spontaneous curvature. The lipid-dependent fusion stage is prior to lipid mixing and fusion pore opening but subsequent to the activation of HA (148), gp120/gp41 (98), and F protein of paramyxovirus SV5 (143).

As in fusion of protein-free lipid bilayers, a stage of biological fusion that depends on the composition of the contacting membrane monolayers is followed by the stage dependent on the distal membrane monolayers. Amphiphiles of positive spontaneous curvature (LPC and chlorpromazine) added to the distal

monolayer of the fusing membranes (the inner monolayer of the cell membrane) promote the opening of a fusion pore in HA-mediated fusion (58, 60, 149) (Figure 4D). Similar promotion has been reported for fusion of rabies virus with liposomes (142). Likewise, secretion from SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins) mutant cells is rescued by LPC added to the outer monolayer of the plasma membrane, which in this case represents the distal monolayer of the fusing membranes (150). Thus, the lipids that facilitate lipid monolayer bending into the edge of a hydrophilic pore (15, 60) promote fusion pore formation in protein-mediated fusion.

To conclude, the effects of nonbilayer lipids on biological fusion suggest that proteins catalyze this process through the same stalk-pore sequence of intermediates as that identified for fusion of protein-free lipid bilayers.

Importantly, lipid inhibitors of fusion such as LPC present a unique and powerful tool for dissecting complex and multistep pathways of biological fusion reactions (98, 143, 151, 152). An LPC block was used in (152) to uncouple the formation of the SNARE complexes in sea urchin egg cortical exocytosis from the membrane fusion event. Similarly, reversible inhibition of membrane merger by LPC, in combination with peptide inhibitors blocking restructuring of fusion proteins into 6-helix bundles, has been elegantly used to demonstrate that fusion mediated by HIV (98) and parainfluenza virus (143) is a result of the transition of the fusion proteins into the bundle configuration and did not occur after this protein restructuring.

4.3.2 LIPIDS IN FISSION Some effects of the lipids on the budding-fission reaction are linked to the recruitment of the proteins involved. For instance, AP-2, part of the clathrin coat machinery, binds to phosphoinositides [reviewed in (52, 132)]. Dynamin binds to acidic lipids (132). DAG binds protein kinase D regulating fission from the *trans*-Golgi network (133). Cholesterol is needed for clathrin coat-mediated budding (153). PE is required for disassembly of contractile ring in cytokinesis (154).

In addition to these specific effects, lipids can directly modulate budding-fission reactions by generating the membrane spontaneous curvature, J_s^B (Section 2.1). Indeed, stimulation of endocytosis by the flippase-dependent translocation of aminolipids from the outer to inner monolayer of the plasma membrane (50, 155) is explained by the developing difference between the areas of the membrane monolayers that, according to the bilayer-couple mechanism (Section 2.1), promotes the spontaneous bending toward the cytoplasm.

The asymmetry in the spontaneous curvatures of the two monolayers is a possible result of action of two acyltransferases implicated in intracellular fission, endophilin (130) and CtBP/BARS (131), which can transform lysophosphatidic acid (LPA) into phosphatidic acid (PA) in the necks of budding vesicles and Golgi tubes. The resulting J_s^B has been suggested to be crucial for fission.

5. MECHANISMS OF MEMBRANE REMODELING

5.1 Hypothetical Intermediates of Biological Fusion

Two radically different pathways of protein-mediated fusion have been suggested. The first proceeds through the entirely proteinaceous fusion pore (Figure 4F,G), while the second starts with membrane hemifusion (Figure 4C). Below we discuss both pathways and consider the specific mechanisms by which proteins can mediate fusion and fission.

5.1.1 PROTEINACEOUS PORE The hypothetical proteinaceous fusion pore establishes an aqueous connection between fusing volumes before any lipid monolayer merger (156, 157). This concept has been strengthened by recent work indicating that the terminal stage in vacuolar fusion in yeast is mediated by the transmembrane complex of two proteolipid hexamers formed by the V0 subunit of vacuolar H⁺-ATPase (157). It has been suggested that these V0 *trans*-complexes establish a proteolipid-lined channel at the fusion site. Ca²⁺-triggered conformational change in proteolipids opens a pore by a radial expansion of the proteolipid oligomeric ring (Figure 4G). Lipid polar heads migrate into the amphiphilic clefts in the lumen of the pores, and hydrocarbon chains of the lipids fill the space between the adjacent proteolipids. The argument for this mechanism boils down to the ability of V0 to form membrane *trans*-complexes and channels that expand in a Ca²⁺/calmodulin-dependent fashion.

This mechanism readily accounts for the earliest fusion stages, in which membranes get into close contact and break their continuity. However, the mechanism addresses neither the force driving the lateral separation of the subunits nor the specific pathway by which a proteinaceous pore transforms into a lipid-involving structure. One might suggest that subunit separation yields two apposing pores, one in each membrane, with edges covered partially by the polar interface of the proteolipid and partially by lipid polar heads. Then the lipidic components of the edges of the adjacent pores merge to establish the continuity of two lipid bilayers. In energy terms, this scenario means that the energy released during separation of the V0 subunits should be sufficient for the formation of a lipid pore edge with energy per unit length of 10⁻¹¹ J/m (Section 2.1). Assuming that each of the V0 subunits has a cross-section radius of ~1 nm, the energy of repulsion between the subunits required for their separation to a distance of 1 nm (the cross-section diameter of a lipid) is ~3k_BT. Protein refolding can provide such energies in the form of steric repulsion only as long as the proteins are adjacent rather than separated. On the other hand, it is hard to identify strong enough long-range repulsive interactions between the subunits required to allow lipids into the pore edge. For instance, electrostatic repulsion between two elementary charges located at the centers of the subunits separated by a 1-nm gap gives only ~0.35k_BT. Even stronger energy requirements have to

be fulfilled to ensure the further expansion of the fusion pore. Hence, the mechanism of fusion through a proteinaceous pore still awaits clarification.

5.1.2 HEMIFUSION INTERMEDIATE The phenomenology of biological fusion is strikingly similar to that of fusion of protein-free bilayers including the lipid dependence, properties of early fusion pores (see above), and the activation energies for membrane rearrangements (31, 158). This suggests that protein-mediated fusion, in analogy to lipid bilayer fusion, proceeds through a hemifusion intermediate (Figure 4C). Although it is still not established that hemifusion is in fact a true intermediate of any biological fusion, the indirect evidence in favor of this hypothesis versus the proteinaceous pore hypothesis is extensive:

1. As discussed above, fusion proteins are indeed able to create hemifusion structures, as has been shown for wild-type and mutant HA and some other fusion proteins [e.g., parainfluenza (76), coronavirus (159), Sendai virus (160), and HIV (161)].
2. HA-expressing cells fusing with planar lipid bilayer demonstrate spreading of lipid dye prior to the opening of an expanding fusion pore (162). Similarly, in an elegant recent study of insulin granule exocytosis, lipid mixing was shown to precede fusion pore opening by ~ 0.3 s (163). Thus, both for viral and for exocytotic fusion, one might observe the sequence of events expected if hemifusion indeed is an intermediate in fusion pore formation. It remains possible, however, that the pore forms outside of the hemifusion structure and independently of it.
3. Proteins that form ionic channels have strict sequence requirements to support both a polar interface with water filling the channel lumen and the hydrophobic interface with a bilayer interior. Thus, the fusion pore formation observed for lipid-anchored HA (63, 164, 165), and the wide range of TM sequences supporting the formation of expanding fusion pores (73) are difficult to reconcile with the hypothesis that HA forms proteinaceous fusion pores and indirectly favor the alternative hypothesis that fusion starts with hemifusion intermediates.
4. LPC in the contacting membrane monolayers blocks fusion at a stage prior to the opening of a fusion pore, showing that the earliest stages of fusion are already dependent on the lipids (98, 148). The dependence of pore formation on the lipids in the distal membrane monolayers also indicates that complete fusion proceeds through lipidic rather than proteinaceous intermediates (58).

To conclude, in our view, there is now a rather solid case for the hypothesis that at least some of the better-characterized reactions of biological fusion, and in particular viral fusion, proceed through hemifusion intermediates similarly to

the fusion of protein-free bilayers. These intermediates, if they indeed exist, are most likely either too small or too labile to be identified by electron microscopy (63).

5.1.3 FUSION PORE AS THE MOST DEMANDING STAGE Assuming that biological fusion, as fusion of protein-free bilayers, starts from local hemifusion and proceeds to the opening of a fusion pore, we can now ask which of the fusion stages is the most energy consuming; in other words, what is the most demanding job for the fusion protein? Experimental results indicate that early fusion stages including the establishment of the close contact and the local merger of the contacting monolayers into a fusion stalk are less demanding than the subsequent opening of a fusion pore and, especially, its expansion. Only at the highest number of activated HAs does fusion reach the stage of an expanding fusion pore (58, 61). Less HA is needed to open a flickering pore, and still less to establish the hemifusion phenotype. Many mutant fusion proteins (56, 76, 87, 159) and fragments of proteins including even short peptides (166, 167) mediate hemifusion but are incapable of forming expanding pores.

These results are consistent with the theoretical analysis above according to which a stalk might require just a few $k_B T$ beyond the energy provided by the thermal fluctuations. The expansion of the HD is already fairly expensive and likely requires the concerted action of multiple fusion proteins (28). Fusion pores are even more expensive. To conclude, the main challenge for proteins likely involves the generation of nonlocal maintainable force that forms an expanding fusion pore.

5.2 Models for Local Rearrangements

Several mechanisms have been suggested to specify the way in which fusion and fission proteins might perform their jobs.

5.2.1 LOCAL MODELS FOR FISSION Most fission mechanisms suggested for the best-characterized dynamin-mediated reaction [reviewed and classified in (168)] can be subdivided into two groups. The first group considers dynamin as a mechanochemical enzyme that changes its conformation upon GTP hydrolysis, resulting in an additional constriction [reviewed in (122)] and/or stretching (169, 170) of the collared tube. Alternative models suggest that dynamin plays the role of a molecular switch (171) that triggers a cascade of downstream reactions mediated by endophilin and leading to fission (129).

A recent mechanism of dynamin-mediated fission reconciles, at least partially, the previous models (172). It proposes that the dynamin helix plays the role of a rigid external skeleton in the membrane neck, imposing constrictions on the neck shape. In these conditions the neck membrane loses its stability and collapses if the dynamin helix stretches and/or tightens as a result of GTP hydrolysis and if the spontaneous curvature of the neck membrane becomes more

negative as a result of the endophilin-mediated reaction (Section 4.3) of conversion of the lipid of positive spontaneous curvature, LPA, to a lipid of almost zero spontaneous curvature, PA. The conformational change in dynamin and the change in lipid composition can act concertedly, making the fission phenomenon more robust.

This model, as well as earlier ideas [reviewed in (173)], describes only the first stages of the fission process, namely constriction of the membrane neck. None of the models address the nucleation of the membrane discontinuity, hemifission (if it exists), and the completion of membrane separation, which are the subject of future work.

5.2.2 LOCAL MODELS FOR FUSION Many suggested fusion mechanisms are based on the assumption that fusion protein restructuring exerts a pulling force on a membrane. The hypothetical fusion machines comprise several protein molecules assembled around the future fusion site. In some models (68, 98, 143, 174, 175), at the first stage of fusion protein restructuring the proteins establish holds on both membranes. In the case of viral fusion proteins such as HA or gp120/gp41 that are anchored in the viral membrane by the TM, this stage involves insertion of the fusion peptide into the target membrane. It has been estimated that binding of the HA fusion peptide to the membrane is strong enough to serve as a reliable hold (84). In the SNARE-pin hypothesis (175), suggested for intracellular fusion proteins, a similar configuration develops upon binding of two integral proteins expressed in different membranes. Further conformational change pulls two membrane anchors of the same protein (or both TMs of the *trans*-complex of the proteins) into close proximity. This stage, best characterized both at the structural and at the functional levels for gp120/gp41 (68, 98) and parainfluenza virus (143), consists of the formation of the outer layer of the 6-helix bundle. This bundle formation directly induces fusion (98). At this stage TMs of the fusion protein can become structural elements of the otherwise lipidic edge of the initial pore (165).

Another hypothetical fusion mechanism suggests that fusion is mediated by activated HAs with their fusion peptides inserted into the viral membrane (84). The subsequent coiled-coil transition in the HA2 ectodomain results in a force pulling the fusion peptides and deforming the viral membrane in the vicinity of the HA trimer. As a result, the trimers self-assemble into ringlike clusters and dimple the viral membrane toward the target membrane. The top of the dimple accumulates the bending stress, which is released when the membranes hemifuse.

A similar mechanism has been suggested for paramyxovirus-induced membrane fusion (95). In this case, fusion peptides are first inserted into the target membrane, and then the fusion protein coiled-coil extends toward the viral rather than target membrane (in the direction opposite that described for HA and gp120/gp41), which dimples the target membrane toward the viral envelope. This kind of scenario was suggested earlier for exocytosis (176). In the scaffold model (176), fusion proteins induce dimpling of the plasma membrane toward the

granule membrane, stress the tip of the dimple, and promote its fusion. Both exocytosis (177) and HA-mediated fusion (178) are inhibited by inflating cells and, thus, by damping of dimple formation by membrane tension. These findings confirm the functional importance of the membrane dimples for fusion.

A recent model suggests that the specific mechanism by which activated HA might form a hydrophobic defect at the top of the dimple is the extraction of the previously inserted fusion peptides (179).

All these models suggest specific protein-driven mechanisms of establishing local intermembrane contact and generating local membrane stresses. Yet none of them address the membrane tension necessary for the most demanding stage—expansion of the fusion pore—because a local protein machine is unable to generate tension in a membrane section larger than the initial fusion site.

5.3 Models for Global Rearrangements

To generate a membrane stress that can drive fusion pore expansion, the fusion machine must act on a larger area of the membrane. Fusion proceeds via stages that are analogous to those of membrane budding-off and fission but in the opposite direction (Figure 5). Therefore, it is tempting to speculate about the force that drives fusion pore expansion on the basis of what is known about the force that drives the constriction of the membrane neck in the budding reaction (180).

Lipid bilayers of membrane buds (Figure 5) in intracellular vesicles and viral particles are often very strongly bent to a radius of curvature as small as 15–20 nm (181, 182). How can proteins bend membranes to the radii, which in protein-free lipid dispersions can be achieved only by using the harshest ultrasound treatments (183)? What drives unbending of these vesicles during fusion?

5.3.1 MODELS BASED ON THE BILAYER-COUPLE HYPOTHESIS A global force driving membrane remodeling may come from interplay of the areas of two membrane monolayers (Section 2.1), which generates the tendency of the membrane to bend for budding or to unbend for fusion. For instance, epsin-induced asymmetry in the monolayer areas apparently drives tubulation of liposomes (125a). However, the bilayer-couple mechanism might work for large membranes only if the absolute number of the protein or lipid molecules inserted into or extracted from one of the membrane monolayers is very large because the action is averaged over the whole monolayer area. Alternatively, the site of fusion or budding/fission represents a small membrane patch mechanically isolated from the remaining membrane by a rigid wall. The stiffness of this wall must greatly exceed the stretching-compression rigidity of a lipid monolayer, so that the tensions generated within the membrane patch do not propagate into the surrounding membrane. To the best of our knowledge, the existence of such rigid walls around fusion or fission sites has never been reported.

In contrast to the large membranes, a small vesicle can be primed to unbending, and hence fusion, by depletion of lipids or proteins from its external

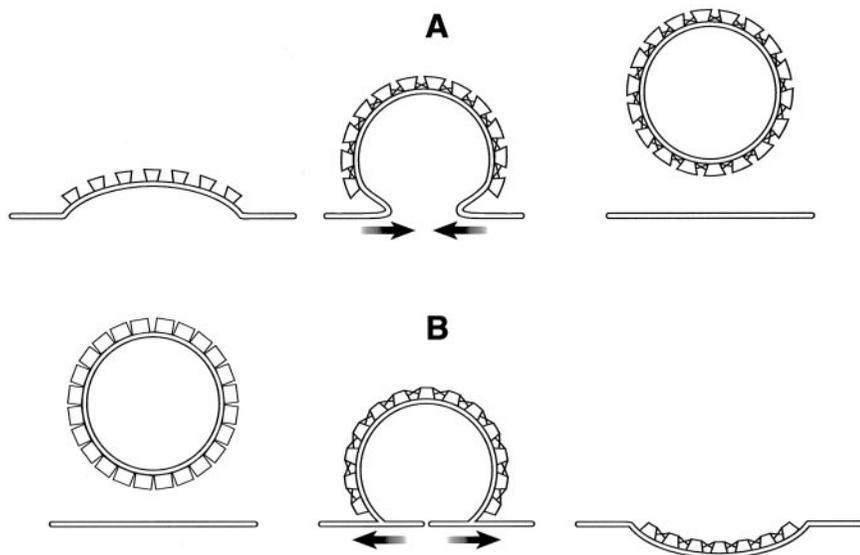


Figure 5 Coat mechanism for membrane remodeling. (A) Budding and fission. (B) Fusion and expansion of the fusion pore.

monolayer. Currently, there is no experimental support for this kind of remodeling mechanism in cells. Budding of protein-free membranes driven by the monolayer area asymmetry results in vesicles, which are much larger than the intracellular vesicles and often remain connected to the parent vesicles (50). In our opinion, the global driving force for membrane remodeling is most likely generated by interconnected protein coats.

5.3.2 PROTEIN COAT MODEL FOR BUDDING To induce budding and fission (Figure 5A), a protein coat has to satisfy two major conditions. First, the attractive interaction between the coat-forming proteins has to be sufficiently strong. The interaction energy has to exceed the bending energy of a membrane fragment of area a covered by one protein, $f_{\text{int}} \geq (1/2)\kappa \cdot a \cdot J^2$. For a bud radius of 15 nm (i.e., a curvature of $J = 2/15 \text{ nm}^{-1}$), an area per protein molecule of $a \approx 100 \text{ nm}^2$, and a bilayer bending rigidity of $\kappa_b \approx 20k_B T$, we obtain $f_{\text{int}} \geq 20k_B T$. This energy is ~ 100 times larger than the energy of the physical interaction between the protein molecules, such as electrostatic attraction and the estimated energy of interaction of clathrin triskelions (184), but it can be provided by a chemical bond.

The second condition relates to the rigidity, κ_p , of the protein coat, which has to exceed the rigidity of the lipid bilayer, κ_b . The bending rigidity of protein layers has never been measured experimentally but was estimated as $\kappa_p = 2000 k_B T$ (180) based on the elasticity of dilatation measured for a number of protein

layers. Accordingly, the dense coat of fusion proteins is likely to be a hundred times more rigid than the underlying lipid bilayer, and the protein coat can, indeed, control the shape of lipid bilayer.

In virus release and in some intracellular reactions, sphere-forming coat proteins such as influenza M1 and COPs might provide the global driving force for membrane budding-fission and also determine the specific pathway of the actual membrane fission. In other reactions, these two functions can involve different proteins. For instance, coats consisting of clathrin, adaptor proteins, and their partners might provide the global force for budding, while dynamin, endophilin, and other cylinder-forming proteins mediate fission.

5.3.3 PROTEIN COAT MODEL FOR FUSION The protein coat model of membrane fusion (180) (Figure 5B) assumes that (a) activated fusion proteins form a dense interconnected protein coat surrounding the zone of intermembrane contact; (b) this coat has a spontaneous curvature opposite to that of the budding coat, and to adopt its preferable configuration, the fusion coat bends out of the initial shape of the membrane surface; and (c) the bending of the protein coat deforms the underlying lipid bilayer and produces tension that drives fusion and expands the fusion pore.

In contrast to the local models, the protein coat model accounts for the force driving the fusion pore expansion until it reaches the dimension of the coat itself. For a virus, whose surface is completely covered by the coat, this means a complete insertion of the viral membrane into the target one.

Some of the features of viral fusion reactions, discussed above, are consistent with this hypothesis. Aggregation of activated fusion proteins (Section 4.1) is crucial for coat formation. The larger the coat, the larger the fusion intermediates that would remain subject to the tension, explaining the observed dependence of the fusion phenotype on the numbers of available fusion proteins (Section 3.2).

In contrast to the local models, the coat hypothesis accounts for the extremely high surface densities of fusion proteins characteristic of many viruses as a factor that drives, rather than sterically hinders, fusion. Furthermore, the protein coat hypothesis is independent of any specific features of fusion proteins, such as the 6-helix bundle motif shared by many but not all viral fusion proteins (67, 70, 71, 185), and can be fairly general.

To the best of our knowledge, the coat hypothesis is the only one suggesting the driving force for all fusion stages from the beginning through the end. However, it remains possible that two functions of the fusion protein, generation of the sustained driving force and local action initiating the reaction, may be separate. For instance, the local action can proceed according to one of the local mechanisms discussed above, while coat assembly drives the later stages. In intracellular fusion, local fusion and pore expansion can be controlled by different proteins. It has also been suggested that the global force that drives the pore expansion is of an osmotic nature [(163), but see (186)].

6. CONCLUDING REMARKS

Does a universal mechanism underlie all reactions of membrane remodeling? Are the remodeling proteins structurally involved in the earliest local intermediates of fusion and fission? Alternatively, do these proteins promote remodeling by priming the contacting bilayers for fusion or fission and then allowing them to reorganize through the sequence of lipid-involving intermediates? Currently, we can answer neither of these fundamental questions unambiguously. However, the available data are consistent with a tempting hypothesis that a universal element in diverse remodeling reactions is rearrangement of lipid bilayer patches involving formation of nonbilayer intermediates. Fusion and fission are then driven by similar but oppositely directed elastic forces, and knowledge accumulated for one of these reactions can be used to better understand another.

If this hypothesis is correct, the direct and local action of the proteins at the contacting monolayers of the merging membranes can be less crucial than commonly assumed. Indeed, in contrast to all known fusion reactions, budding and fission are often mediated by proteins, which are located at the distal membrane monolayers and thus cannot directly interact with the contacting monolayers. Therefore, any similarity between the mechanisms by which proteins merge bilayers in fusion and fission has to be related to the way in which proteins, present on either of the two sides of the membrane, shape and stress it.

This hypothesis is clearly too simple to fully describe biological reactions. Although it gives some idea of the possible forces involved in membrane rearrangements and the related requirements for protein action, it leaves open the question of the structure of the specific protein machinery that releases the conformational energy of the proteins and delivers it to the intermediates of membrane fusion and fission. Future studies will, hopefully, bring together detailed characterizations of different protein machines, functional studies describing the pathways of membrane rearrangements, and theoretical analysis of configurations and energies of the intermediate structures of membrane remodeling to uncover the answers to the fascinating question of how proteins merge and divide lipid bilayers.

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