Membrane Fusion

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Common themes are emerging from the study of viral, cell-cell, intracellular, and liposome fusion. Viral and cellular membrane fusion events are mediated by fusion proteins or fusion machines. Viral fusion proteins share important characteristics, notably a fusion peptide within a transmembrane-anchored polypeptide chain. At least one protein involved in a cell-cell fusion reaction resembles viral fusion proteins. Components of intracellular fusion machines are utilized in multiple membrane trafficking events and are conserved through evolution. Fusion pores develop during viral and intracellular fusion events suggesting similar mechanisms for many, if not all, fusion events.

Membrane fusion is a ubiquitous cell biological process (*1*). Fusion events that mediate housekeeping functions—endocytosis, constitutive secretion, and recycling of membrane components—occur continuously in all eukaryotic cells. Additional fusion events occur in specialized cells—intracellularly, as in regulated exocytosis of hormones, enzymes, and neurotransmitters, as well as intercellularly, as in sperm-egg fusion and myoblast fusion. Fusion events are also associated with disease states: the formation of giant cells during inflammatory reactions, the entry of all enveloped viruses into host cells, and, in the case of the human immunodeficiency virus (HIV) for example, virally induced cell-cell fusion, which leads to cell death.

It is useful to categorize fusion reactions topologically. In many fusion events, the leaflets that face the cytoplasm make the initial contact; this category encompasses fusion of intracellular macromolecular carrier vesicles with their target organelles. In contrast, for cell-cell and virus-cell fusion the exoplasmic leaflets, those that face the external milieu, make the initial contact.

Influenza HA-Mediated Membrane Fusion

The protein responsible for influenza virus fusion is its well-characterized hemagglutinin (HA) (*2*). A combination of factors has contributed to the prominence of HA-mediated fusion as a model membrane fusion system (*2, 3*): (i) A single gene product, the HA protein, confers fusion activity. (ii) Fusion can be rapidly and synchronously triggered by exposing HA-containing membranes to low pH; in an infection, this happens in endosomes. (iii) A soluble oligomeric HA ectodomain can be readily produced for biochemical and biophysical analyses of steps preparatory to fusion. (iv) The x-ray structure of the neutral pH (prefusogenic) form of the HA ectodomain is known (*4*). (v) A large database of cloned HA gene sequences exists. (vi) Numerous mutants (spontaneous, selected, and engineered) with altered fusion phenotypes have been characterized (*2, 3, 5–7*).

The three-dimensional structure of the HA ectodomain has allowed interpretation of results of experiments designed to elucidate the fusion mechanism of HA (*4*). Several aspects of this structure are highlighted in Fig. 1. HA is a trimer of three identical subunits, each of which contains a fusion peptide, a conserved sequence containing many hydrophobic amino acids. In the neutral pH structure, the three fusion peptides, one per monomer, are located in the fibrous stem of the molecule, about 30 Å away from where the protein enters the viral membrane (Fig. 1A). The fusion peptides are tightly tucked into the subunit interface (Fig. 1B) by a network of hydrogen bonds. They are crucial to HA-mediated fusion because mutations within the fusion peptide alter (*5*) or abolish (*6*) fusion activity, and because the fusion peptides must be released from the molecular interior for fusion to proceed (*8, 9*). In addition to its role in fusion, HA is also responsible for the initial binding interaction between the virus and the target cell (*2*). The three receptor binding sites, one per monomer, lie at the distal tips of the globular head domains (*4*), approximately 100 Å away from the fusion peptides (Fig. 1A). Although influenza can fuse efficiently with membranes that lack receptors (*10*), binding of the virus to receptors accelerates steps prior to fusion (*11*).

How do the fusion peptides, which are neatly held in place about 100 Å away from the top of the trimer, aid in destabilizing the viral and target bilayers, a prelude to fusion? Recent experimentation has shed light on this important question. In response to low pH, the tertiary structure of HA is altered (*2, 11–14*). Very rapidly, the fusion peptides, and other sequences buried

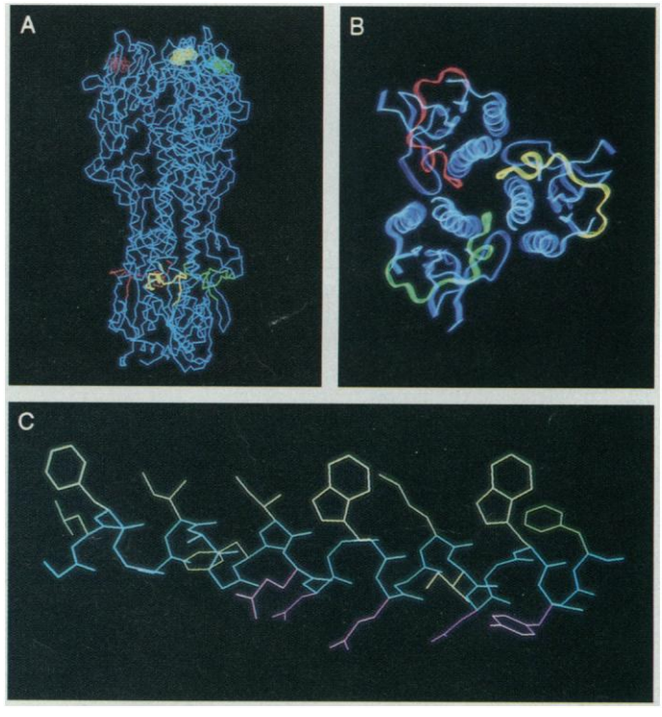

Fig. 1. The influenza hemagglutinin. (**A**) The α carbon backbone (blue) of the HA trimer is displayed with the three fusion peptides, located in the stem of the molecule, highlighted (red, yellow, green). Molecular surfaces of sialic acid residues are shown (red, yellow, green) in the receptor binding sites of the globular head domains. (**B**) Ribbon diagram of the HA trimer (cross section) highlighting the three fusion peptides. (**C**) A two-dimensional projection of the fusion peptide, HA2 residues 1 to 24, as an α helix. Side chains of residues with hydrophobicity indices (*53*) ≥0.64 (Ile, Phe, Leu, Trp, Met) are shown in yellow; side chains of residues with hydrophobicity indices ≤0.26 (Glu, Asn, Asp, Tyr) are shown in purple.

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in the stem, are exposed (*14*) and changes occur at the distal tips of the head domains, most likely reflecting their partial separation (*9*). Second, the globular heads dissociate substantially from one another, as revealed by epitope exposure (*14*) and by electron microscopy (*15*). The rapid initial changes are clearly required. If the head domains are physically joined, exposure of the fusion peptides and fusion are severely and commensurately impaired (*8, 9*). Complete dissociation of the globular head domains may, however, not be required (*14, 15*). Rather, the fusogenic conformation is envisioned as a transient intermediate along the pathway of changes induced by low pH; it is thought to be a trimer with exposed fusion peptides that is only modestly altered in its globular head domains (*2, 9, 11, 15*).

The exposed fusion peptides render the HA ectodomain hydrophobic and foster its immediate attachment to the target membrane (*16*) before (*17*) significant lipid mixing occurs. After exposure of the fusion peptides and their binding to the target membrane, there is a lag (*11, 17–19*), the length of which depends on HA surface density, pH, temperature, and the presence of a receptor in the target membrane (*11*). The lag phase likely involves additional conformational changes as well as rotational and lateral motions of HA trimers in the plane of the viral membrane (*20*). The net result is thought to be aggregation of several HA trimers (*11, 18, 21*) and formation of a fusion pore within the interior of the aggregate (Fig. 2, A and B). Electrophysiological (*19*) and electron microscopic (*22*) studies have provided strong support for the existence of an HA fusion pore.

Exocytosis of Mast Cell Granules

Regulated exocytosis involves the fusion of an intracellular storage vesicle with the cytoplasmic face of the plasma membrane with concomitant expulsion of the vesicle contents. Fusion occurs in response to an external stimulus that often triggers increases in cytoplasmic Ca^{2+} or other second messengers. As in the case of the influenza HA, knowledge of the fusion trigger, or its proximal precursors, has allowed experimental synchronization of the fusion event (*23*). Many systems are used for studying regulated exocytosis—the sperm acrosome reaction, oocyte cortical granule exocytosis, secretory vesicle fusion in a variety of endocrine and exocrine cells, and neurotransmitter release (*24*). I will focus on exocytosis of mast cell granules, because this system has been central in recent years in the development of the concept of a fusion pore.

Powerful studies of mast cell exocytosis have combined the use of patch-clamp technology with mast cells from a mutant mouse, the beige mouse, that have extraordinarily large secretory vesicles (1 to 5 μm in diameter). Because membrane capacitance is strictly proportional to membrane surface area, fusions can be monitored as increases in membrane capacitance, provided that the donor membrane, in this case the secretory granule, is sufficiently large. By patch clamping the plasma membrane, stepwise capacitance increases can be seen that reflect the fusion of successive secretory granules (*25–27*). As in other exocytic fusion systems, neither Ca^{2+} nor adenosine triphosphate (ATP) appears to be directly required for fusion (*23, 24, 28, 29*). Nor, as had previously been thought, is vesicle swelling (*30*).

The electrophysiological events that accompany fusion of giant mast cell granules can be summarized as follows: The first indication of fusion is an outward current transient that marks the discharge of the vesicle's membrane potential through a nascent fusion pore. A few milliseconds later, the capacitance of the mast cell plasma membrane increases due to incorporation of new membrane surface from the secretory granule. During this time a narrow, electrically conducting connection, the fusion pore, forms between the secretory vesicle and the outside world. The pore widens over the next hundreds of milliseconds, during which time the capacitance grows to a new plateau level (*26*).

High-resolution electrophysiological analysis (*26*) has been used to examine the exocytic fusion pore. Pore opening occurs in two stages, an initial abrupt opening followed by a gradual dilation. The initial pore is estimated to be 2 to 2.5 nm in diameter and there is usually one pore per vesicle. Initial pore opening is reversible; a single vesicle can attempt to fuse several times before it succeeds. The initial pore conductance is variable and grows at variable rates. Although there is conduction through the pore, its formation does not cause general membrane leakiness.

Intra-Golgi Transport

Membrane fusion is essential for the movement of macromolecules between intracellular organelles, the latter process often being referred to as membrane trafficking. An extensively characterized membrane trafficking event is transport within the Golgi apparatus (*31–34*). Advances with this system have come from the pioneering studies of J. Rothman and his colleagues who first set up a biochemical assay to monitor the fusion-dependent transport of protein from one compartment of the Golgi

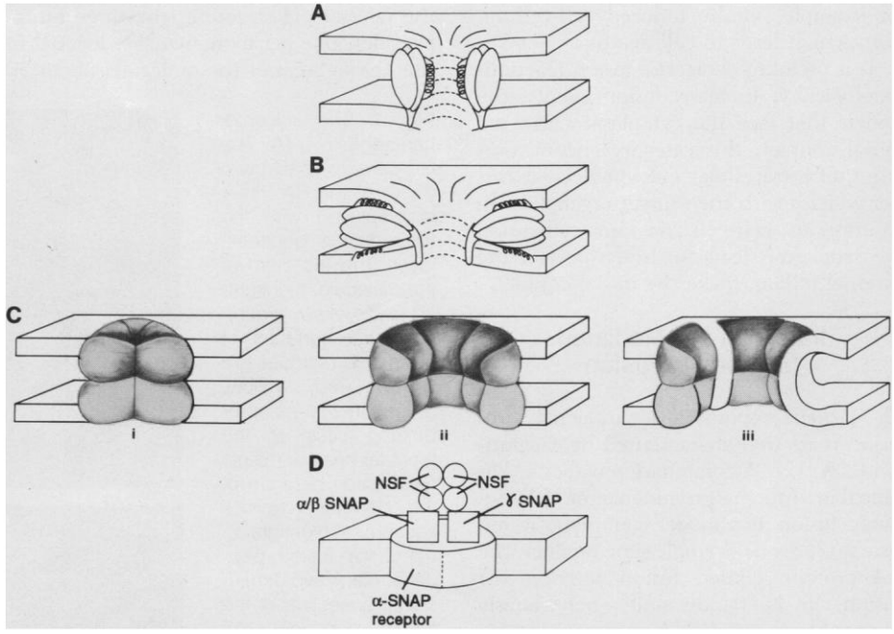


Fig. 2. Models of fusion pores and a fusion machine. (**A**) A model for an HA fusion pore lined with several upright HA trimers. The exposed fusion peptides projecting into the pore are thought to promote lipid mixing (*21, 51, 52*). (**B**) A model for an HA fusion pore lined with several tilted HA trimers. The exposed fusion peptides are thought to bind to both the viral and target membranes, bringing them into close apposition (*11*). (**C**) A model for the exocytic fusion pore formed by paired integral membrane multimeric proteins in the vesicle and plasma membranes (*49, 59*). (i) The pore is closed. (ii) The pore opens. (iii) The pore dilates. (**D**) Minimal components of the NSF-containing fusion machine (*32, 45, 46*). It is not yet known whether γ-SNAP binds to the α-SNAP receptor or to a different molecule.

Table 1. Comparison of the fusion pores that form during influenza HA-mediated fusion and mast cell degranulation. Data are compiled from (*19*) and (*26*). GTPδS, guanosine-5′-*O*-(3-thiotriphosphate).

System	Experimental trigger	Delay before fusion (s)	Mean initial conductance (pS)	Mean rise time (μs)	Estimated initial pore diameter (nm)	Flicker	Leaky to ions	Dilation time (s)
HA pore	H^+	~20–200	150	135	1–2	Yes	No	~100
Mast cell pore	GTPδS	~10–20	285	100	2–3	Yes	No	~0.2

to the next. By the careful application of inhibitors, these investigators have synchronized the transport process, thereby allowing its dissection. Intra-Golgi transport has been reconstituted in fused cells, in a cell-free system with isolated Golgi membranes, and, recently, in semi-intact cells (*32–34*). Complementation of the biochemical analysis with genetic studies in yeast (*35–37*) and with electron microscopy (*38*) has been very informative.

The steps (*32*) involved in transporting material between Golgi cisterna can be summarized as follows: (i) A nascent (nonclathrin) coated bud forms on the donor cisterna. (ii) The coated transport vesicle detaches. (iii) The coated transport vesicle is targeted to and attaches to the acceptor cisterna. (iv) The transport vesicle is uncoated. (v) The uncoated vesicle, attached to the acceptor cisterna, matures. (vi) The mature uncoated vesicle fuses with the acceptor cisterna depositing its contents (*39*). This is clearly a very complex process that must require many proteins for its occurrence, specificity, and regulation. Mutations in 12 genes disrupt the related transport step between the endoplasmic reticulum (ER) and the Golgi complex in the yeast *Saccharomyces cervisiae* (*37*). The requirements for nucleotides, cofactors, and certain regulatory proteins along the pathway have been defined (*32*). ATP and guanosine triphosphate (GTP) are required for vesicle budding and fusion. GTP hydrolysis is required for uncoating. Fatty acyl coA is required for both budding and maturation (*32*). Ca^{2+} is required, most likely as a cofactor, late in transport (*34*). Small monomeric GTPases (for example, members of the Arf and Rab families), large trimeric GTPases, and phosphoproteins regulate the process, most likely to ensure its fidelity, timing, and vectorial nature (*40*).

A complex of three proteins has emerged as the core of the "fusion machine" that assembles at the interface between the transport vesicle and the acceptor cisterna (*41*). The assembly contains a homotetramer called NSF [NEM (*N*-ethylmaleimide)–sensitive factor/fusion protein] (*42*), which attaches by means of a set of peripheral proteins, α-, β-, and γ-SNAP (soluble NSF attachment protein) (*43, 44*) to an integral membrane receptor of the acceptor cisterna (*45*). A 35-kD integral membrane protein, the α-SNAP receptor, has recently been identified as a component of this receptor (*46*).

NSF was the first component of the fusion machine to be characterized molecularly (*42*). Each 76-kD subunit consists of three domains, two ATP-binding domains, and a third domain of unknown function. NSF is conserved through evolution (*32*); its equivalent in *Saccharomyces cerevisiae* is sec18p, a protein essential for the yeast secretory pathway (*42*). NSF is used for several membrane trafficking events: ER to Golgi transport, constitutive fusion of Golgi-derived vesicles with the plasma membrane, and endosome-endosome fusion (*47*). NSF-related proteins may mediate other intracellular fusion events (*48*). NSF thus appears to be a widely distributed, widely used, and highly conserved element of the fusion machine. A model has been put forward in which ATP hydrolysis, which causes dissociation of NSF from the membrane-bound SNAPs, drives the fusion process (*32*).

Fusion Pores

Fusion pores have been detected and analyzed in detail with high-resolution electrophysiological techniques in two different fusion events, regulated exocytosis of mast cell granules, in which membrane leaflets facing the cytoplasm make initial contact, and fusion mediated by the influenza HA, in which membrane leaflets facing the extracellular milieu make initial contact. Although differences exist in the preparatory and completion stages of pore formation, the two pores are quite similar (Table 1) in terms of conductances, estimated diameters, opening times (rise time), lack of leakiness to ions, and capacity to close and reopen (flicker). The pore that forms between an HA-expressing fibroblast and a red blood cell (*18*), however, takes longer than the exocytic pore to form and to dilate (Table 1). The longer delay time for the onset of this HA-mediated fusion event may reflect the need for several HA trimers to aggregate to form the pore (*11, 18, 19, 21*). This is in contrast to exocytic fusion for which a preassembled pore has been invoked to account for the rapidity of fusion in response to external stimulation, especially in the case of synaptic transmission (*49*). The longer dilation time for the observed HA pore may reflect special constraints imposed by the red cell cytoskeleton or differences in the lipid or protein rearrangements that must occur to complete HA-mediated fusion. Recent studies combining patch-clamp analysis and image-intensified fluorescence microscopy suggest that the HA fusion pore opens before there is substantial lipid mixing (*50*). This result suggests that if the initial HA pore contains lipid, as implied in most models (see below), these lipids are relatively immobile, and significant lipid mixing first occurs during pore dilation.

In HA-mediated fusion, we know from electrophysiological studies that a pore forms and from biochemical, molecular biological, and structural studies that HA is the fusion protein, that HA contains a fusion peptide, and that several trimers appear to be required. Two models (*11, 17, 21, 51, 52*) that incorporate these ideas are shown in Fig. 2, A and B. The models share the following important features: (i) An aggregate of several modestly altered HA trimers circumscribes an incipient pore of small diameter; (ii) fusion initiates in the interior of this aggregate; (iii) the fusion peptides act as α helices (*51, 53*); (iv) the fusion peptides interact with lipid components of both the viral and the target membranes; and (v) fusion proceeds through a nonbilayer intermediate (*54*). The models differ on two interrelated points. In model A (Fig. 2A), the HA trimer remains upright and presents the hydrophobic faces of several fusion peptides to the interior of the pore where they serve a wetting function (*52*), facilitating the mingling of lipids from the viral and target membranes. Bentz and colleagues suggest that this occurs via an inverted micelle (*52*), an intermediate in certain liposome fusion systems (*55*). In model B (Fig. 2B), the HA trimer bends such that the fusion peptides bind to the outer (exoplasmic) leaflets of the viral and target bilayers. This binding interaction or the close membrane approach that it fosters (or both) may induce a nonbilayer structure in the interior of the HA aggregate. Next a pore, the first connection between the viral and target cell interiors, opens. There are difficult questions posed by both models including,

for model A, whether it is feasible for a few fusion peptides of relatively small hydrophobic surface area to provide a wetting surface, and, for model B, the difficulty in conceiving asymmetric bending of a trimer. Interesting refinements and variants of these models have been proposed. One (56) invokes specific interactions between transmembrane domains of adjacent trimers in facilitating HA tilting and in forming the pore. Another related variant (57) invokes a "lipid-stalk" (58) as the nonbilayer intermediate.

In the case of regulated exocytosis, we know, from electrophysiological studies, that a fusion pore forms (*25–27, 30*). We do not yet know, however, its molecular constituents, let alone their important biochemical properties. Nonetheless, several models for the exocytic fusion pore have been described. In a model proposed by Almers (59), the exocytic fusion pore develops in three steps: (i) insertion of a multimeric fusion protein, an integral resident of the granule membrane, into the apposed plasma membrane; (ii) a conformational change producing an opening in the center of the multimer; and (iii) dilation of the pore by dispersal of the multimeric subunits and concomitant incorporation of lipid molecules at the former subunit interfaces. A variant of this model, suggested by Almers and Tse (49), is shown in Fig. 2C: two multimeric hemiproteins, one in the granule and one in the plasma membrane, bring the two about-to-be-fused membranes into close apposition, in analogy to the paired rings of subunits that constitute gap junction channels (59). The variant model obviates the high energetic cost, inherent in the original model, of inserting a large multimeric, and presumably hydrophilic, ectodomain into the apposed bilayer. (It also makes it easier to envision the later stages of pore dilation.) The second and third steps of the model (Fig. 2C) correlate with the two phases of growth of the pore conductance as dissected by high-resolution patch-clamp analysis (26). An assumption of the model is that the exposed subunit interfaces (Fig. 2C, step iii) can provide an appropriately amphipathic surface to foster the proposed flow of lipids that leads to fusion.

The model put forward by Almers and co-workers (49, 59) presents a simple image with which to conceptualize a fusion pore (Fig. 2C). However, two aspects of the model—that the initial pore is purely proteinaceous and that exocytic fusion does not involve nonbilayer lipid structures—are being actively discussed. Two other models have recently been proposed that address these issues, one by Zimmerberg and colleagues (60), which considers the initial pore to be composed of both proteins and lipids, and one by Fernandez and colleagues (61), which suggests that the initial pore is purely lipidic with proteins serving as an external scaffold. Despite their important differences, all three models for regulated exocytosis propose an aggregate of fusion-promoting proteins circumscribing a pore of small initial diameter as the first connection between the vesicle interior and the outside world.

Fusion pores have been documented during other regulated secretory events by transmission and quick-freeze electron microscopy and by patch-clamp analysis (29, 59, 62). In addition, indirect evidence suggests that other viral fusion proteins may aggregate and form pores under fusion-inducing conditions (63). Thus, protein-mediated, or at least facilitated, pore formation seems to be a common theme for viral and cellular fusion events. It is not yet known whether the constituents of the NSF-containing fusion machine (Fig. 2D) organize into a pore-forming structure. Future experimental work addressing the validity of various models for the HA and exocytic fusion pores should reveal the fundamental similarities and differences in the ways in which fusion pores assemble, the precise structures of their protein and lipid components, and the ways in which they dilate. These two examples should be particularly useful because the HA and exocytic pores exemplify ones that initiate fusion reactions between exoplasmic leaflets for HA and cytoplasmic leaflets for exocytosis.

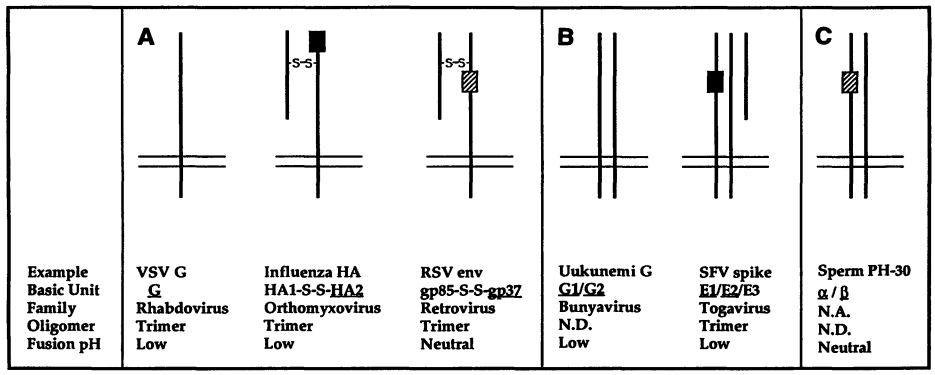
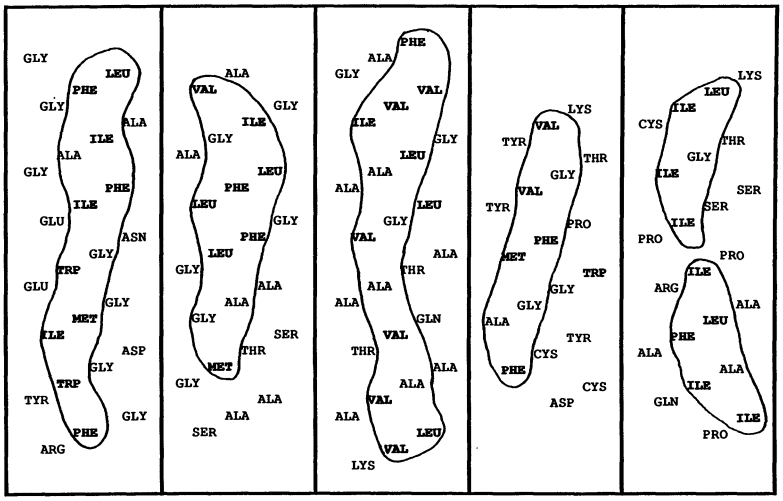


Fig. 3. Viral fusion proteins and a candidate cell-cell fusion protein. (**A**) Viral fusion proteins with a basic unit of one type I integral membrane protein. Other proteins that fall in this category are those of the paramyxovirus, flavivirus, and coronavirus families. (**B**) Viral fusion proteins with a basic unit of two type I integral membrane proteins. (**C**) Topological organization of a putative sperm fusion protein. Membrane-anchored subunits are underlined. Known fusion peptides, those supported at least by mutagenesis, are shown as solid black boxes. Potential fusion peptides, the best candidate within the complex (*51, 53, 68*), are shown in hatched boxes. Putative internal fusion peptides have been identified (*80*) in both VSV G (*75*) and in the Uukunemi G. For the influenza HA, the Rous sarcoma virus (RSV) envelope (env), and the Semliki forest virus (SFV) spike, the non–membrane-anchored subunits arise from proteolytic cleavage of membrane-anchored precursors (HA0, pr95, and p62, respectively). The two subunits of PH-30 arise from separate larger precursors (*68*). The fusion proteins of more complex viruses (for example, herpes viruses) may not fit into these simple motifs (*65*). In some cases, rearrangements of viral spike oligomers may be necessary for fusion (*65–67*). N.D., not determined; N.A., not applicable.

Fusion Proteins and Fusion Machines

A working premise is that fusion is an energetically unfavorable event and that proteins aid in overcoming the repulsive hydration force that discourages fusion or in promoting the hydrophobic attractive force that favors fusion (64). Influenza-mediated fusion is enacted by a fusion protein, the integral membrane HA glycoprotein (Figs. 1A and 2, A and B). Hypothetical fusion proteins have been incorporated into models (for example, Fig. 2C) for the exocytic fusion pore (59, 60). Intra-Golgi transport is mediated by a proteinaceous multisubunit fusion machine (*32, 41*). What generalizations can we make about fusion proteins and fusion machines?

The fusion proteins of enveloped viruses are the simplest characterized to date (*2, 65*). In most cases, a single gene product, the fusion protein, is necessary and sufficient to confer fusion activity when expressed in a tissue culture cell or when reconstituted into a liposome. The basic unit of most viral fusion proteins is one (Fig. 3A) or two (Fig. 3B) type 1 integral membrane glycoproteins. These often combine into trimers (for example, Fig. 1A) or tetramers that project from the viral envelope. In many cases one of the integral membrane proteins is made as a precursor, and cleavage is necessary for optimal fusion activity under physiological conditions. In most cases, the fusion protein is also responsible for binding the virus to its host



Protein	Influenza HA2	HIV gp41	SV5 F1	SFV E1	PH-30 α
Fusion pH	low	neutral	neutral	low	neutral
Location	amino terminal	amino terminal	amino terminal	internal	internal
Residues	23	18	26	16	21
Average H.I.	0.5	0.7	0.7	0.6	0.5
H.I. hydrophobic face	1.1	0.9	1.0	0.9	1.2

Fig. 4. Characteristics of the fusion peptides of several viral fusion proteins and a potential fusion peptide from a candidate sperm fusion protein. The sequences analyzed encompass those from the first to the last residue of the hydrophobic face (encircled residues) of the displayed helices. Bulky hydrophobic residues (H.I. ≥0.64; Ile, Phe, Val, Leu, Trp, Met) are in bold. Hydrophobicity indices (H.I.) were calculated with the normalized consensus scale of Eisenberg (*53*). Helices were plotted by using the program HELO from R. Stroud.

cell receptor, although the binding function may reside in a distinct protein subunit or domain (Fig. 1A). The key feature of most viral fusion proteins is a fusion peptide, a stretch of relatively hydrophobic amino acids within a membrane-anchored subunit (Figs. 1, 3, and 4). Different viral fusion proteins promote fusion at either neutral or at mildly acidic pH (Figs. 3 and 4). As discussed above, when HA encounters low pH, its fusion peptides are exposed, thereby rendering the HA ectodomain hydrophobic and able to interact with a target bilayer. Conformational changes have been documented in other viral fusion proteins (*66, 67*), and a common theme, triggered exposure of fusion peptide residues resulting in conversion of the fusion protein ectodomain into a hydrophobic entity, has been suggested for several viral fusion proteins (*65–67*). In the case of viral fusion proteins that function at neutral pH, a major challenge is to identify the presumed trigger.

Can we extrapolate and use viral fusion proteins as paradigms for those that mediate other fusion reactions initiated between exoplasmic leaflets, that is, those that promote cell-cell fusion events? Preliminary evidence from our laboratory suggests that we can. A sperm surface antigen with a role in fusion with the egg, PH-30, resembles viral fusion proteins in many striking respects (*68*). Like togavirus and bunyavirus fusion proteins, PH-30 is a complex of two type I integral membrane glycoproteins (Fig. 3, B and C). Both PH-30 subunits are made as larger precursors, and the final processing cleavage correlates with the acquisition of fertilization competence. As PH-30 contains an integrin ligand domain, it is likely to be responsible, at least in part, for binding sperm to the egg plasma membrane. Thus, like most viral fusion proteins (Figs. 1A and 3, A and B), PH-30 may be a dual functional complex, aiding both binding and fusion to the target membrane. Finally, PH-30 contains a sequence that shares characteristics with viral fusion peptides (Figs. 3 and 4). Future experimentation will reveal whether PH-30 is necessary and sufficient for fusion and whether its putative fusion peptide is a bona fide one.

Viral fusion proteins will most likely only serve in a limited sense as prototypes for proteins that promote fusion events that initiate between cytoplasmic leaflets, for example those that promote exocytosis. This is because proteins that face the extracellular environment and those that face the cytoplasm differ in their biochemical properties; the exoplasmic and cytoplasmic leaflets of biological membranes differ in their compositions and physical properties (*69*); and the needs for and mechanisms available to regulate fusion events that initiate between exoplasmic or cytoplasmic leaflets differ. Nevertheless, it is tantalizing that independently derived models for the exocytic fusion pore (Fig. 2C) and the HA fusion pore (Fig. 2, A and B) invoke a ring of integral membrane protein subunits that change conformation and expose a hydrophobic moiety (*11, 17, 21, 51, 52, 56, 57, 59, 60*). This analogy should be considered with caution, however, until the components of the exocytic fusion pore are molecularly defined and until firmer evidence is provided for a ring of (how many?) HA trimers at the fusion site.

How shall we define a fusion protein? Given that membrane fusion entails the merger of two lipid bilayers, given that the fusion proteins of enveloped viruses are all integral membrane proteins, and given that we have recently gained a heightened appreciation for the role of the HA transmembrane domain in its fusion mechanism (*70*), it is reasonable to propose that integral membrane proteins are key players in fusion reactions. We can therefore invoke an operational definition of a fusion protein as an integral membrane protein that, upon trigger, changes conformation so as to expose a hydrophobic domain that promotes mixing of lipid components from two apposed bilayers.

In view of the preceding operational definition, it is important to consider that fusion proteins and fusion machines may share similarities. The concept of a fusion machine first proposed by Rothman and his co-workers (*41*) is an appealing one. It connotes a complex of integral and peripheral proteins that is necessary and sufficient to promote fusion with the target bilayer. The minimal intra-Golgi fusion machine is depicted in Fig. 2D. The following lines of evidence suggest that other proteins may embellish the fusion machine. In addition to sec18p (NSF) and sec17p (α-SNAP), a third secretory gene product that is predicted to be a membrane protein, sec22p, is required for fusion of ER-derived vesicles with the Golgi apparatus in yeast (*35, 37*); sec22p may therefore be involved in intra-Golgi fusion as well. Several peripheral proteins, in addition to NSF, crosslink to α-SNAP in the fusion machine (*46*). A peripheral homo-oligomer, p115, has recently been shown to be required for Golgi transport in vitro (*71*).

It is not yet clear which components of the fusion machine are critical for membrane merger, in other words, for fusion per se. Although early attention focused on NSF, the finding that the NSF homolog in yeast, sec18p, is required for binding ER-derived vesicles to Golgi membranes (*37*), coupled with its position in the complex (Fig. 2D), suggests that the role of NSF may be to foster a tight binding interaction between the donor and target membranes,

an obligate prerequisite to fusion; NSF may also function at a later stage of the overall fusion-dependent transport pathway (*32*). Given the preceding discussion about the known or potential importance of integral membrane proteins in fusion reactions, the integral membrane NSF-SNAP receptor could be a key player. In view of current models for fusion pores (Fig. 2, A through C), it will be interesting to determine if the α-SNAP receptor (*46*) functions as a single subunit or as a homo- or hetero-oligomer and if the core components of the fusion machine (Fig. 2D) ever exist in higher order assemblies.

Reiterating themes from the virus literature, Rothman and Orci have proposed (*32*) that the NSF-containing fusion machine undergoes a conformational change that exposes a proteinaceous hydrophobic domain that promotes the merger of lipids from the two fusing bilayers. If this hypothesis proves true, it would suggest a fundamental similarity between a fusion protein and a fusion machine. It is still formally possible, however, that an integral membrane fusion protein, as operational defined above and perhaps as part of a fusion machine, provides this function. Alternative possibilities exist as well (*61*).

Is regulated exocytosis accomplished by a fusion protein or a fusion machine? The hypothetical integral membrane multimer in the Almers model for the exocytic fusion pore (*49, 59*) would, by my operational definition, be called a fusion protein. However, this hypothetical exocytic fusion protein may not function alone (*36, 72, 73*): (i) Constitutive exocytosis in yeast requires ten gene products for its regulation and execution. (ii) Similarly, at least 13 gene products are involved in the pathway of regulated exocytosis in eukaryotic ciliates. (iii) A multisubunit complex has been detected in synaptic vesicles that may participate in fusion. (iv) As discussed in the accompanying article by Creutz and elsewhere, peripheral annexin proteins, perhaps in conjunction with fatty acids, other Ca^{2+}-binding proteins, phosphoproteins, GTPases, and cytoskeletal elements may participate in exocytic fusion. If the hypothetical integral membrane protein that promotes exocytic fusion (Fig. 2C) requires other peripheral components, then it would be more appropriate to state that regulated exocytosis is carried out by a fusion machine rather than a fusion protein. As with any fusion machine, a major challenge would still be to identify the critical bilayer destabilizing and fusion-promoting elements. Recent progress in reconstituting a regulated secretory event (*74*), coupled with genetic systems for evaluating regulated exocytosis (*73*), should aid in the identification and assignment of functions to proteins that participate in exocytic fusion.

Fusion Peptides

A combination of biochemical, biophysical, and molecular biological studies has established amino acids 1 to 24 of the HA2 subunit (Fig. 1) as the fusion peptide of the influenza HA (*2, 51*). Modeling exercises have suggested potential fusion peptides in other viral fusion proteins, and several of these assignments have been supported by site-specific mutagenesis (*75*). Fusion peptide sequences have the following properties (*51*): They are short (16 to 26 amino acids) and relatively hydrophobic (hydrophobicity index = 0.5 to 0.7). If modeled as α helices (Figs. 1C and 4), they display one face with a high hydrophobicity index (≥0.9) and a back face that has hydrogen bonding potential. Fusion peptides are always in a membrane-anchored subunit, although they can be located at the NH_2-terminus or internal to the polypeptide chain. Some potential internal fusion peptides have prolines near their centers. This is reminiscent of melittin, a small (26 amino acids) membrane-interactive protein in which a central proline kinks an amphipathic α helix (*76*). Fusion peptides are highly conserved within but not between virus families. There do not seem to be major features that distinguish the fusion peptides from proteins that function at neutral or low pH (Fig. 4).

Several lines of evidence indicate that the fusion peptide of the influenza HA, either as the isolated peptide or in the context of the soluble HA ectodomain, interacts with target bilayers as an α helix (Fig. 1C) (*53*). Although the fusion peptide in the soluble HA ectodomain, which is completely free to rotate in solution, is thought to lie in a roughly parallel fashion (*16*) along the surface of a bilayer (Fig. 2B), a recent study from Helenius and co-workers suggests (*17*) that the fusion peptide in the context of membrane-anchored HA interacts in a more oblique fashion, as has been proposed for other viral fusion peptides (*77*). At present, it is thought that the HA fusion peptide does not fully traverse the bilayer in a perpendicular fashion (*13, 16*), as has been proposed for lytic pore-forming amphipathic peptides (*78*); it would be difficult to envision an internal fusion peptide traversing a bilayer.

Putative fusion peptides have been seen in other viral fusion proteins and in one candidate cell-cell fusion protein, PH-30 (Figs. 3 and 4). Do we expect to find fusion peptides in proteins that participate in other fusion reactions? Not necessarily. Neither NSF nor α-SNAP, the only components of the intra-Golgi fusion machine sequenced to date, contain a putative fusion peptide. If fusion peptides cannot be found in a fusion protein or among the minimal components of a fusion machine, then it may be that a hydrophobic tertiary structural element, a "fusion patch" (*79*), provides the equivalent function.

In summary, the themes of fusion pores, fusion proteins and machines, and fusion peptides and patches are reverberating in the current literature in three well-characterized fusion systems. The idea of a highly localized fusion event, most likely involving fusion pores that are circumscribed with proteins, is gaining momentum. Detailed analyses are now essential to determine the extent to which the mechanisms of the HA, cell-cell fusion proteins, the exocytic fusion pore, and the NSF-containing fusion machine are similar. On the basis of topological considerations, the mechanisms of viral and cell-cell fusion proteins will likely be most related to each other, and, similarly, the mechanisms of regulated exocytosis, intra-Golgi transport, and other intracellular vesicle fusion events will likely be most related to one another. The fusion pore may be the common element that unites all of these cellular fusion events.

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The Annexins and Exocytosis

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The annexins are a group of homologous proteins that bind phospholipids in the presence of calcium. They may provide a major pathway for communication between cellular membranes and their cytoplasmic environment. Annexins have a characteristic "bivalent" activity in the sense that they can draw two membranes together when activated by calcium. This has led to the hypothesis that certain members of this protein family may initiate contact and fusion between a secretory vesicle membrane and the plasma membrane during the process of exocytosis.

Introduction

Membranes compartmentalize cells and isolate them from their immediate environment. The process of exocytosis is one of the major routes by which this isolation is broken. Secretory products, including small molecules as well as complex proteins, are sequestered in membrane-bound secretory vesicles. The membranes of these vesicles may then fuse with the cell surface membrane, releasing the contents of the vesicles, which may be messengers, such as insulin or epinephrine, or laborers, such as invertase or trypsin, or building blocks, such as collagen or proteoglycan.

A complete understanding of the process of exocytosis requires knowledge of the molecular events comprising vesicle formation, vesicle translocation, vesicle fusion, and membrane recovery by endocytosis. As this knowledge is gained, our general understanding of membrane structure and regulation will advance in parallel. The study of exocytosis may be the beneficiary of advances in other areas of molecular cytology, or it may be a guiding beacon for other fields of exploration. Most probably, it will be both.

In the late 1970s, application of the traditional "grind and find" approach of the biochemist to the problem of membrane fusion in exocytosis led to the identification of synexin (*1*), a protein that catalyzes secretory vesicle membrane contact and fusion in vitro. It was hypothesized that synexin might act at the point of fusion of secretory vesicles with the plasma membrane, as well as between fusing vesicles in compound exocytosis. Subsequently, it was found that synexin is but a single representative of a class of homologous proteins, the annexins (*2*), that bind to lipid membranes in a calcium-dependent manner. Members of this group of proteins may be involved more widely in intracellular membrane trafficking, as well as in the regulation of a diverse array of calcium-dependent events on membrane surfaces (*2*). Because of the newly recognized breadth of the annexin family and the explosion of hypotheses for annexin functions, their involvement in exocytosis has recently received less singular attention. However, the apparent versatility of this protein family should not detract from recognition of its potential importance in exocytosis. In this article, the "annexin hypothesis" for the process of exocytosis will be re-evaluated in the context of recent advances in the study of membranes, annexins, and exocytosis.

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Membrane Fusion Mediated by the Annexins

In common with the prototype, synexin (now also referred to as annexin VII), most of the annexins are able to promote the calcium-dependent aggregation of isolated secretory vesicles. Conversely, all proteins that have been found to exhibit a similar activity have proven, on the basis of primary structure, to be members of the annexin family. Synexin was originally isolated as the active principle that promoted adrenal medullary chromaffin granule aggregation when the granules were incubated with crude cytosolic extracts (*1*). Synexin appeared to act as a glue in this process, rather than as an enzyme that catalyzed changes in the membrane surface, as the synexin was found to bind to the chromaffin granules at 5 to 10 μM calcium (*3*). However, half-maximal amounts of chromaffin granule aggregation by synexin required