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MINI-REVIEW

Membrane Fusion of Enveloped Viruses: Especially a Matter of Proteins

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Abstract

To infect mammalian cells, enveloped viruses have to deposit their nucleocapsids into the cytoplasm of a host cell. Membrane fusion represents a key element in this entry mechanism. The fusion activity resides in specific, virally encoded membrane glycoproteins. Some molecular properties of these fusion proteins will be briefly described. These properties will then be correlated to the ability of a virus to fuse with target membranes, and to induce cell–cell fusion. Some molecular and physical parameters affecting virus fusion—at the level of either viral or target membrane or both—and the significance of modelling virus fusion by using synthetic peptides resembling viral fusion peptides, will also be discussed.

Key Words: Viruses; fusion proteins; membrane fusion; endosome; protein conformation; membrane dehydration; cell fusion; synthetic peptides.

"For when was honey ever made
with one bee in the hive"
(Thomas Hood, in *The Last Man*)

Introduction

The above quotation bears general validity, but appears to be particularly applicable when evaluating recent progress in understanding the molecular details of membrane fusion. Early observations by electron microscopy showed that chemically induced fusion of cells was accompanied by an extensive redistribution of intramembrane particles, causing the formation of

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protein-devoid areas, often referred to as "bare lipid patches." Proteins were considered as a barrier for membrane merging and, consequently, it was proposed that fusion resulted from direct lipid–lipid interactions between such patches in apposed cells (Ahkong *et al.*, 1975). Indeed, the concept of fusion requiring bare lipid patch formation was strengthened by observations of biophysicists and biochemists that pure lipid vesicles of appropriate compositions could rapidly fuse, provided that a fusogenic agent was included in the medium (Papahadjopoulos *et al.*, 1978). However, that proteins could actually act as a fusogen became apparent when virologists and cell biologists noted that animal viruses were capable of inducing fusion between cells and that this event was very much dependent on the presence of certain virus-specific proteins (Poste and Pasternak, 1978; White *et al.*, 1983). Similarly, exocytotic events in secretory cells also require proteins to mediate the fusion between secretory vesicles and the plasma membrane, i.e., proteins are not cleared from the fusion site during early events as revealed by electron microscopy, using ultrafast freezing techniques (Schmidt *et al.*, 1983; Plattner *et al.*, 1988). More recently, evidence has been presented that protein-induced fusion is also intimately involved in processes by which newly synthesized proteins reach their various cellular destinations after synthesis and assembly in the endoplasmic reticulum is completed (Wilson *et al.*, 1989). Interestingly, detailed studies (Balch *et al.*, 1984) of the synthesis of a *viral* protein and of its subsequent transfer between Golgi cisternae, mediated by vesicular transport and membrane fusion, paved the way that led to the identification of an intracellular protein, possibly acting as a universal intracellular fusion protein in the endocytic and biosynthetic pathways in eukaryotic cells (Pfeffer and Rothman, 1987; Wilson *et al.*, 1989; Diaz *et al.*, 1989).

In many respects viruses play a pivotal role in revealing and analyzing a variety of fundamental processes in cell biology, including the synthesis and processing of membrane proteins, intracellular sorting, and the intracellular flow and fusion of membranes. In this review the discussion will be limited to some aspects of their membrane fusion properties. Several characteristics and properties of the viral (membrane) proteins will be described, as they are of great significance in understanding virus–cell and virus-induced cell–cell fusion at the molecular level. A section is included on virus-induced fusion between cells, and the relevance of such studies in understanding mechanisms of fusion in general, and virus fusion in particular, is discussed. Subsequently, a variety of parameters governing viral fusion will be described. Some attention will be paid to the role of the target membrane in virus fusion, specifically with respect to the role of cholesterol. The significance of using synthetic peptides, resembling part of the sequence of viral fusion proteins, in revealing mechanistic details of virus fusion will be discussed. By necessity, many of the issues discussed here will be described briefly. For more detailed discussion

of certain topics the reader is referred to a variety of reviews that have been published in recent years (White *et al.*, 1983; Marsh, 1984; Düzgüneş, 1985; Kielian and Helenius, 1986; Wilschut and Hoekstra, 1986; Wiley and Skehel, 1987; Spear, 1987; Ohnishi, 1988; Hoekstra and Wilschut, 1989; Compans *et al.*, 1989; Hoekstra and Kok, 1989).

Structural, Molecular, and Biological Features of Animal Viruses

The structural elements of animal viruses comprise a membrane or "envelope," which consists of lipids derived from the plasma membrane of the host cell on which the virus grows, and one or more virally encoded glycoproteins. A matrix protein, M, is located just underneath the viral membrane. Via their cytoplasmic tails, the transmembrane envelope proteins may interact with the matrix protein (Dubovi and Wagner, 1977; Lamb and Choppin, 1983), as has been demonstrated, for example, by cross-linking studies and immunocytochemical staining (Dubovi and Wagner, 1977; Ohno and Ohtake, 1987). Presumably, such interactions will restrict the motional freedom of the viral envelope proteins, a significant parameter in expressing viral fusion activity, as will be discussed below.

The envelope surrounds the viral nucleocapsid, which consists of the genome and capsid proteins, arranged in a regular helical or icosahedral structure (Fuller, 1987).

The envelope glycoproteins protrude ca. 100–150 Å from the virus bilayer and can be readily recognized by electron microscopy, given their characteristic size and shape. They consist of a long fibrous stem, topped by a globular domain. The envelope proteins are commonly referred to as "spikes." For most viruses discussed in this paper, the protein composition of the envelope is extremely simple as it may contain as few as one or two types of proteins.

Viral replication can only be initiated when the particle manages to deliver its nucleocapsid into the cytoplasm of a host cell. To do so the capsid has to cross two barriers, the viral membrane and the plasma membrane of the host cell. This crossing is accomplished by membrane fusion (Fig. 1). Some viruses fuse directly with the plasma membrane, at neutral pH. This pathway is used by viruses belonging to the family of paramyxoviruses [e.g., Sendai virus, Newcastle disease virus, Simian virus 5 (SV5) and measles virus]. Most other viruses, including myxoviruses (e.g., influenza virus), toga viruses (e.g., Semliki Forest virus, SFV), and rhabdoviruses (e.g., vesicular stomatitis virus, VSV) enter the cell by receptor-mediated endocytosis, followed by fusion in the endosomal compartment, triggered by a mild acidic pH. It is interesting to note that depending on the virus involved, the

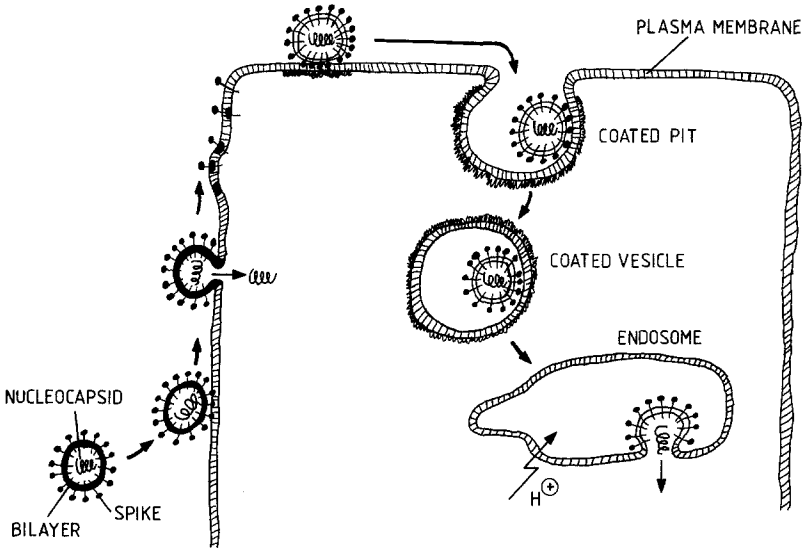

Fig. 1. Entry mechanisms of enveloped viruses into mammalian cells. Depending on the family to which a virus belongs (see text), entry can occur via fusion with the plasma membrane (left), at neutral pH, or by internalization via receptor-mediated endocytosis (top) followed by fusion with the endosomal membrane "from within." The latter process is triggered by a mild-acidic pH.

threshold pH values at which fusion is triggered in the latter entry pathway may vary between approximately 5.0 and 6.5. Recently, it has become apparent that distinct subpopulations of endosomes exist that can be distinguished, among others, with respect to their acidification properties (Schmid *et al.*, 1988). The so-called "early endosomes" are less acidic than the "late" endosomes. Thus, it may well be possible that the intracellular site of virus penetration can now be further differentiated and may depend on the pH at which the virus acquires fusion competence (see also below).

It has been firmly established that specific viral envelope proteins are responsible for bringing about the fusion reaction. The most compelling evidence has been provided by selectively expressing, in eukaryotic cells, viral proteins from cloned copies of their genes, inserted into recombinant vectors. Thus far, it has been demonstrated that fusion activity resides in one type of protein only—as revealed by susceptibility of the cells to fuse—and that expression of the fusion activity does not require other viral proteins (White *et al.*, 1982a; Kondor-Koch *et al.*, 1983; Florkiewicz and Rose, 1984; Sambrook *et al.*, 1985; Paterson *et al.*, 1985; McCune *et al.*, 1988; Boulay *et al.*, 1988). The exclusive localization of virus fusion activity in one type of glycoprotein is probably the case for most viruses. Yet, herpes simplex virus

seems to be an exception, as current evidence suggests that fusion of this virus results from a concerted action of several membrane glycoproteins (Spear *et al.*, 1989). In Tables I and II some molecular and structural properties of several of the best-characterized viral fusion proteins are summarized.

When mammalian cells are infected with a virus the host cell's protein synthesis is shut off, except for synthesis of viral proteins. This propensity has enabled a detailed understanding of the synthesis, assembly, and intracellular transport of the envelope proteins, and consequently deepened the insight of how the viral fusion protein structure is related to its functioning (see, e.g., Boulay *et al.*, 1988; see also below). In general, the synthesis of viral proteins can be seen as a valuable model for understanding the processing of membrane and secretory proteins (Pfeffer and Rothman, 1987).

For protein synthesis to occur, the nucleocapsid has to be released into the cytoplasm. The mechanisms involved in nucleocapsid uncoating and subsequent events occurring prior to and during early replication are still largely obscure. Recent work concerning replication of SFV RNA suggests that it takes place while the capsid is still closely associated with cytoplasmic organelles (Strauss and Strauss, 1986). These organelles, earlier referred to as "cytopathic vacuoles," appear to be endosomes and lysosomes, modified by virus-specific components (Froshauer *et al.*, 1988). Via morphologically distinct structures (filamentous and granular networks) at the cytoplasmic surface of these vacuoles—in which the viral RNA polymerase is located —connections are formed between the vacuoles and the rough endoplasmic reticulum. Consequently, it has been proposed (Froshauer *et al.*, 1988) that "cytopathic vacuoles" constitute the site of RNA replication in the cytoplasm of the infected cell. Hence, endosomes and lysosomes may serve as a matrix for toga virus RNA replication. Since penetration of these viruses occurs at the level of the endosome, these results imply that the nucleocapsid is not *released* into the cytoplasmic space but, rather, remains associated with the fusion site, i.e., at the surface of the endosomal membrane, and, when these vesicles proceed along the endocytic pathway, remains associated with that of the lysosome.

The structural properties of the viral membrane proteins are critical to their proper intracellular processing and their functioning as a fusogenic protein. Isolated viral proteins or improperly processed (e.g., truncated) proteins, which are secreted into the medium (Florkiewicz and Rose, 1984; Sambrook *et al.*, 1985), are nonfusogenic. Nevertheless, many structural aspects of several of these proteins have been resolved by studying the "solubilized" proteins, obtained by proteolytic cleavage of the spike's ectodomain. The most appealing example is the spike of influenza, HA, which can be removed by proteolysis with bromelain, causing the cleaving off of the hydrophobic membrane anchoring segment that is located in the

Table I. Some Viral Fusion Glycoproteins

Virus	Virus family	Glycoprotein[a]	Molecular weight (kDa) and structure	pH Dependence	References
Sendai virus	Paramyxovirus	$\underline{F_1} + F_2$	52 + 11 homotetramer	neutral[b]	Blumberg *et al.*, 1985 Sechoy *et al.*, 1987
Influenza virus	Orthomyxovirus	$HA_1 + \underline{HA_2}$	44 + 30 homotrimer	5.1–5.8	Wiley and Skehel, 1987
Semliki Forest virus (SFV)	Toga- or Alpha-virus	$\underline{E_1}, E_2 + E_3$	51, 52 + 11 heterononamer	$\leqslant 6.2$	Schlesinger and Schlesinger, 1986
Vesicular Stomatitus virus (VSV)	Rhabdovirus	$\underline{G}$	61 homotrimer	< 6.2	Rose and Gallione, 1981 Kreis and Lodish, 1986
Human immuno-deficiency virus (HIV)	Retrovirus	gp(120) + $\underline{gp(41)}$	120 + 41 ?	neutral[b]	Starcich *et al.*, 1986 Kowalski *et al.*, 1987 Gallaher, 1987

[a]The proteins that are derived from a common precursor and cleaved during processing are linked by a '+.' The peptide containing the hydrophobic fusion sequence is underlined.

[b]"Neutral" indicates that the optimal fusion activity is centered around pH 7.4. The exact pH dependence of HIV fusion is controversial (see text).

Table II. Hydrophobic Segments of Some Viral Fusion Glycoproteins[a]

Virus/fusion peptide	Sequence
	1 5 10 15 20 25 30
Sendai virus/F_1	N-Phe Phe Gly Ala Val Ile Gly Thr Ile Ala Leu Gly Val Gly Val Ala Thr Ser Ala Gln Ile Thr Ala Gly Ile Ala Leu Ala Glu Ala
Influenza/HA_2	N-Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr Gly Tyr His
VSV/G	N-Lys Phe Thr Ile Val Phe Pro His Asn Gln Lys Gly Asn Trp Lys Asn Val Pro Ser Asn Tyr His Tyr Cys Pro Ser Ser Ser Asp Leu
HIV/gp41	N-Ala Val Gly Ala Ile Gly Ala Met Phe Leu Gly Phe Leu Gly Ala Ala Gly Ser Thr Met Gly Ala Ala Ser Leu
	80 85 90 95 100 105 110
SFV/E_1	-Lys Val Tyr Thr Gly Val Tyr Pro Phe Met Trp Gly Gly Ala Tyr Cys Phe Cys Asp Ser Glu Asn Thr Gln Leu Ser Glu Ala Tyr Val Asp Arg

[a]Sequences have been presented by Blumberg *et al.*, 1985 (F_1), Gething *et al.*, 1980 (HA_2, strain A/Japan/305/57), Rose and Gallione, 1981 (G), Starcich *et al.*, 1986 (gp41, strain WMJ-1), and Garoff *et al.*, 1980 (E_1). It should be noted that strain-dependent differences in sequence exist.

COOH-terminal region of the HA_2 subunit (Brand and Skehel, 1972). The three-dimensional structure of the water-soluble ectodomain (called BHA) has been determined to a 3 Å resolution (Wilson *et al.*, 1981)—as yet, the only viral envelope protein of which the 3-D structure is known.

The structural organization of the spike proteins of influenza (HA), VSV (G), and SFV (E) has been best characterized thus far. For both HA (Wilson *et al.*, 1981; Wiley and Skehel, 1987) and G (Dubovi and Wagner, 1977; Kreis and Lodish, 1986) a homotrimeric structure has been established. Trimerization occurs rapidly (1–3 min) after synthesis in the endoplasmic reticulum and is essential for proper transport to the Golgi complex (Gething *et al.*, 1986a; Copeland *et al.*, 1986). Noncovalent interactions between the monomeric subunits stabilize the spike structure, particularly at the stem and transmembrane regions of the spike (Kreis and Lodish, 1986; Doms *et al.*, 1986). The amino acid sequence of regions involved in non-covalent intersubunit interactions appears to be highly conserved between virus strains within a given subtype (Air and Laver, 1986; Wiley and Skehel, 1987). The relevance of this conservation for viral membrane protein structure has been elegantly demonstrated by a double infection procedure with influenza viruses containing variant HA subunits (Boulay *et al.*, 1988). Stable, functionally active hybrid trimers are obtained only when there exists a substantial degree of sequence compatibility between the variant subunits, particularly for those amino acids that are thought to be involved in stabilizing the noncovalent intersubunit interactions.

The structural arrangement of the fusion protein of SFV, E, is also trimeric. Each subunit by itself is a heterotrimer consisting of three glycopeptides, E_1, E_2, and E_3. E_1 and E_2 are transmembrane proteins while E_3 is a peripheral protein (Schlesinger and Schlesinger, 1986). The closely related Sindbis virus does not contain the E_3 polypeptide (Welch and Sefton, 1979), suggesting that this subunit does not play a role in fusion. Intersubunit stabilization is likely located in the transmembrane regions of E_1/E_2 since monomers, rather than trimers (cf. influenza HA, see above) are obtained when the ectodomain is isolated by proteinase K digestion (Kielian and Helenius, 1985).

Recent evidence, obtained by chemical cross-linking techniques, suggests that the fusion protein of Sendai virus, F, is also oligomeric. The oligomers are possibly arranged as tetramers, consisting of two identical dimers (Sechoy *et al.*, 1987; see also Hoekstra and Kok, 1989).

Examination of the viral fusion proteins at the molecular level has revealed that some, but not all, of these proteins are synthesized, assembled, and transported as inactive precursors that need to be activated by proteolytic cleavage before they acquire fusion capacity. This proteolytic cleavage, which does not cause the loss of a (cleaved) polypeptide segment, occurs near

or at the cell surface and is mediated by a host cell enzyme. Artificial activation can be accomplished by briefly treating the (inactive) virus or the expressed viral fusion proteins at the surface of cells that do not contain, or somehow cannot express, the required proteolytic activity, with low concentrations of trypsin. Examples of viruses that require activation are influenza ($HA_0 \rightarrow HA$), Sendai and other paramyoxyviruses ($F_0 \rightarrow F$), and human immunodeficiency virus (HIV, gp160 → gp120 + gp41). Both the G protein of VSV and E_1 of SFV, in which the fusion activity resides, are typical examples of uncleaved proteins. It should be noted that the situation for the SFV E protein is slightly more complex. E_2 and E_3 are initially synthesized as a precursor, p62, which is, in fact, proteolytically processed after passage through the trans Golgi network (Schlesinger and Schlesinger, 1986). Within the Golgi complex the pH is in the order of 6.0–6.5 whereas the threshold pH for *wt*SFV fusion is about 6.2. It is possible, therefore, to envision a protective role of p62 for E_1, preventing the viral fusion protein from becoming activated at an inappropriate site and prior to viral assembly. Hence, E_1 needs to be activated, but the mechanism obviously differs from the activation mechanism noted for influenza, Sendai virus, and HIV.

Activation of the viral fusion proteins by proteolytic cleavage results in the formation of a new N-terminal end. Each monomeric subunit thus actually consists of two polypeptides (e.g., HA_1 and HA_2 for influenza viruses and F_1 and F_2 for paramyxoviruses) which are usually linked to one another via a disulfide bridge (White *et al.*, 1983; Tables I and II). A comparison of the sequence of the N-terminal regions in various virus families (most notably in paramyxo- and myxoviruses) indicated that these regions (20–30 amino acids on length) were exceptionally hydrophobic and, moreover, highly conserved (cf. Table II; Blumberg *et al.*, 1985; Starcich *et al.*, 1986; Wiley and Skehel, 1987; Gallaher, 1987). In addition, site-specific mutations in this region severely modifies the fusion activity of influenza HA (Gething *et al.*, 1986b). These arguments make it reasonable to assume that these hydrophobic segments are highly significant for virus fusion. It would thus appear that proteolytic cleavage and the concomitant formation of an N-terminal hydrophobic domain are essential elements in viral fusion protein activation. In this respect E_1 (SFV) and G (VSV) are exceptions and do not fit this criterion as both proteins do not require proteolytic cleavage and, interestingly, neither one contains an outspoken hydrophobic N-terminal region. It has been proposed, however, that in E_1 an internal uncharged stretch of 17 residues, located about 80 amino acids from the N-terminal, might act as the putative fusogenic domain (Garoff *et al.*, 1980; Dalgarno *et al.*, 1983). This proposal is mainly supported by the notion that this particular region is highly conserved among different strains, but biochemical evidence is lacking. Similarly, the N-terminus of G is highly conserved in different VSV

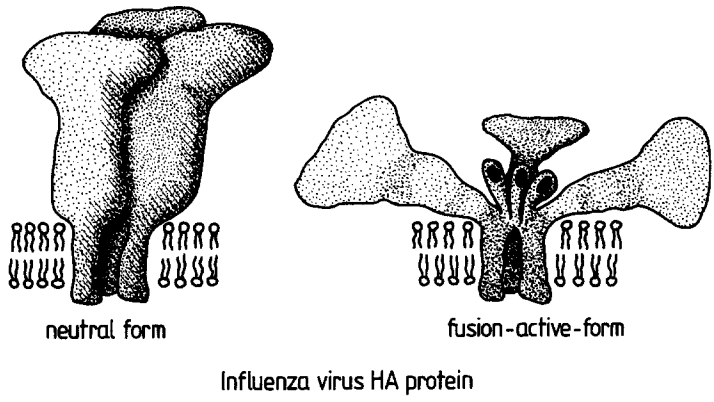

Fig. 2. The mild-acidic pH-induced conformational change in influenza HA. At neutral pH the hydrophobic N-terminae of HA_2 (dark areas, right) are concealed within the trimeric structure of the spike. Binding activity is located in HA_1 at the top of the globular HA structure. Mild-acidic pH causes a partial dissociation of the ectodomain of the trimeric structure. As a result, the hydrophobic region of HA_2 becomes exposed and is thought to interact with the target membrane, thus triggering the fusion reaction (cf. Fig. 4).

strains (Kotwal *et al.*, 1983). However, also in this case a direct involvement of this segment in viral fusion has yet to be demonstrated.

Using isolated viral membrane proteins (i.e., the proteolytically cleaved ectodomains) and intact viruses, evidence has been obtained that the hydrophobic sequences contained in the fusion proteins are either exposed, i.e., for those viruses that fuse at neutral pH (e.g., paramyxoviruses; Hsu *et al.*, 1981) or become exposed by a conformational change in the fusion protein upon a brief exposure to mild acidic pH (Fig. 2). The latter applies to those viruses that enter the cell by receptor-mediated endocytosis, followed by fusion in the endosomes. The acid-induced conformational change in the fusion protein of influenza (Wiley and Skehel, 1987) and SFV (Schlesinger and Schlesinger, 1986) is irreversible, whereas that in the G protein of VSV appears to be reversible (Crimmins *et al.*, 1983). The occurrence of these structural transitions has been most convincingly demonstrated by employing monoclonal antibodies (mAb), specific for the acid or neutral conformations (Daniels *et al.*, 1983; Webster *et al.*, 1983; Copeland *et al.*, 1986; Kielian and Sayad, 1990). Furthermore, the low pH-induced conformational change of these fusion proteins appears to be a carefully regulated process as opposed to denaturation, since the acidic forms maintain a defined structure, as revealed by an (intrinsic) fluorescence study (Wharton *et al.*, 1988), while recognition of the acidic structure by acid-specific mAb's is abolished upon denaturation (Kielian and Sayad, 1990).

For infectious entry into a cell the virus has to bind first to the cell surface. Many virions that enter the cell by receptor-mediated endocytosis

contain binding activity in the protein that also contains fusion activity, though both functions reside at different sites of the molecule (cf. Fig. 2). By contrast, paramyxoviruses, which fuse at neutral pH, contain a separate binding protein, HN. The binding activity of HIV is located in the gp120 polypeptide of the gp160 protein (Kowalski *et al.*, 1987). Thus binding and fusion activity reside in the same molecule in this case, in spite of the claim that HIV fuses at neutral pH (Stein *et al.*, 1987; McClure *et al.*, 1988). It is possible, however, that HIV may penetrate cells by endocytosis as well (Pauza and Price, 1988). Determination of the relative contribution of each pathway, at defined conditions, may clarify at least part of the existing controversy.

After binding (paramyxoviruses) and endocytosis (myxo-, toga- or rhabdoviruses) fusion is triggered. This is thought to be accomplished by the hydrophobic domain in the fusion protein as a result of its capacity to penetrate into the target membrane, which eventually brings about the merging between viral and target membrane.

Systems and Methods of Studying Virus Fusion

To investigate the fusogenic properties of viruses, a variety of experimental systems are available. They include: virus-induced fusion between cells ("*fusion from without*"); the direct monitoring of fusion of viruses with cells or artificial membranes; or fusion of cells that express viral proteins at their surface as accomplished by infecting cells with whole virus or by using vectors that contain specific viral protein genes. With the latter systems, fusion between cells may be induced in confluent cell cultures ("*fusion from within*"), or the interaction between infected and noninfected cells or liposomes can be studied.

A battery of methods is used to detect fusion. The ability of a virus to cause hemolysis of erythrocytes is one of the simplest means to do so. Cell–cell fusion can be detected by phase-contrast microscopy, though early, local fusion events go unnoticed by this procedure. More recently, a number of more direct procedures have been developed that monitor either the mixing of lipids during virus–target membrane fusion or, as applicable in certain systems, the mixing of aqueous contents. Lipid mixing procedures are based on using spin-labeled or fluorescently tagged lipids, which are incorporated into the viral membrane or a target membrane that fuses with cells, expressing viral proteins (Lyles and Landsberger, 1979; Hoekstra *et al.*, 1984; Loyter *et al.*, 1988; Ohnishi, 1988; Hoekstra, 1990). These approaches make it possible to monitor fusion directly and continuously, such that the *initial* interaction events, relevant to understanding the mechanism of virus fusion,

can be studied. In addition, with these kinetic assays it has been possible to simulate virus membrane fusion by using a mass action kinetic model. This model allows one to distinguish between the distinct steps in the overall fusion reaction, i.e., binding of a virus to a target membrane and the actual fusion step itself. By thus determining the aggregation and fusion rate constants at varying conditions, parameters affecting either step can be analyzed (Nir *et al.*, 1986a,b; Hoekstra *et al.*, 1989; Hoekstra, 1990). Mixing of aqueous contents has a limited application and has been mainly used in systems involving erythrocyte ghosts. Fluorescently labeled proteins (Sekiguchi *et al.*, 1981) or cytochemical stains (Doxsey *et al.*, 1985) can be entrapped in ghosts and the depositing into nonlabeled cells upon virally mediated fusion can then be detected. A lucifer-yellow microinjection method has been used to detect early events, i.e., local fusion, during virus-induced cell–cell fusion (Kempf *et al.*, 1987). In the case of studies with artificial membranes it is possible to entrap RNAse in liposomes and to monitor fusion by degradation of viral RNA (White and Helenius, 1980). Advantages and disadvantages of these and other procedures have been described and discussed in detail elsewhere (Hoekstra, 1990).

Virus-Induced Cell–Cell Fusion: "From Without" and "From Within"

Virus-induced fusion between cells is not a common physiological event, as it requires appropriate conditions to induce this process. Much of the earlier work on virus fusion activity involved defining and characterizing conditions under which viruses caused cell fusion (Poste and Pasternak, 1978). It can be accomplished by two mechanisms (Fig. 3) called "fusion from without" (FFWO) and "fusion from within" (FFWI). FFWO takes place upon addition of a relatively large viral dose to the cells. Fusion is triggered within minutes when, after virus-mediated cell aggregation at low temperature, the temperature is raised to 37°C and when the virus fusion protein has been activated, if necessary, by brief exposure to mild acidic pH. FFWI is a late cell–cell fusion event, occurring when a relatively low dose of viruses is used to infect a cell. Thus, FFWI requires viral replication and eventual insertion and exposure of the fusion protein at the cell surface. This implies that for cell–cell fusion to occur, *intact* virus particles are *not* required. On the other hand, as noted above, isolated ectodomains of viral fusion proteins do not induce cell–cell fusion when added to cell cultures. This implies that, among others, proper anchoring and, presumably, exposure of the proteins are relevant parameters in determining fusion activity.

The susceptibility and efficiency of cell–cell fusion depends on the viral strain and cell culture conditions (Poste and Pasternak, 1978; Spear, 1987).

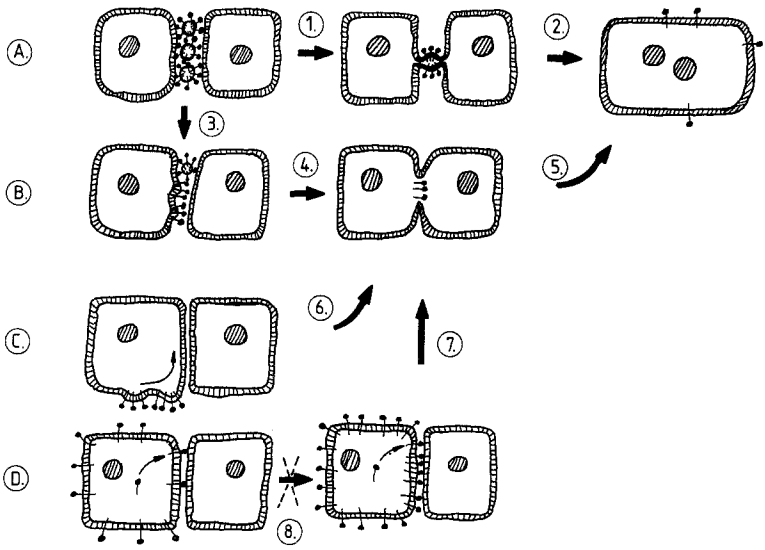

Fig. 3. Mechanisms of viral protein-induced cell fusion; FFWO and FFWI. (A) The "bridge concept." Adjacent cells are aggregated by virus particles. By simultaneous fusion of a virus particle with the two apposed membranes, a cytoplasmic bridge is formed (1). Osmotic cell swelling causes the fusion site to expand, resulting in a dikaryon (2). Only the latter step is recognized by phase-contrast microscopy. (B) Alternatively, after virus-induced cell aggregation, individual virions may fuse with one of the apposed membranes (3). Subsequently membrane-inserted viral proteins trigger the fusion with the apposed membrane (4; see Fig. 4). After local cell–cell fusion the cells swell as in (A), resulting in formation of a dikaryon (5). According to this model virus–cell and cell–cell fusion are separate events. (C) This model depicts the possible migration of viral fusion proteins, inserted into the membrane by virus–cell fusion, into the contact zone between adjacent cells. At sufficiently high local density the proteins may then trigger local cell fusion (6). (D) Cell–cell fusion induced "from within." Two possibilities are considered. Left: the amount of viral protein expressed at the cell surface is relatively low. If the proteins could migrate into the contact area between the (cultured) cells, a local density might be attained high enough to trigger the fusion reaction (8, 7). This is analogous to the model in (C). Right: a relatively high amount of viral protein is expressed at the cell surface. The cells fuse locally (7), eventually resulting in dikaryon formation (5). As described in the text, (C) and (D), left, are highly unlikely mechanisms for cell fusion. Note that in (D), right, only the cell at the left expressed viral membrane proteins. This suffices to trigger FFWI, as discussed.

The temperature and pH are important for both FFWO and FFWI to occur, the former being related to the fusion reaction itself while the latter is needed for the viral fusion protein to be converted to and/or maintained in the fusion-active conformation.

Virus-Induced Fusion "From Without"

Sendai virus has been most extensively used to study cell–cell fusion "from without." In this respect, one of the first observations was reported by

Okada (1958) who demonstrated that Sendai virus, at a relatively high input multiplicity, caused the fusion of Ehrlich's ascites tumor cells at neutral pH.

Fusion requires the close approach of adjacent cells. This is accomplished by the virus' ability to cross-link the cells by attachment of the binding protein HN to sialic acid receptors on the cells' surfaces. Virus-induced cell aggregation is accomplished by an incubation of the system at low temperatures (4–10°C). Under these conditions fusion between the envelope and the plasma membrane does not occur. Furthermore, molecular alterations in the structural organization of the plasma membrane during virus binding are not observed (Knutton, 1978). A rise in temperature (> 20°C) causes the cells to fuse, which appear as large swollen polykaryons after some 15–30 min at 37°C. This sequence of events occurs when so-called "late harvest" Sendai virus is used to trigger fusion. However, with "early harvest" Sendai virus (i.e., viruses isolated from embryonated eggs 24 h postinfection, as opposed to 72 h for "late harvest" virions), no apparent swollen polykaryon formation could be detected, in spite of the fact that fusion between viral envelope and plasma membrane took place normally (Hosaka and Shimizu, 1977). Electron-microscopic studies revealed that small cytoplasmic bridges had formed between the cells (Fig. 3; Knutton, 1978, 1979). These sites could be expanded upon exposure of the system to a hypotonic medium, resulting in the distinct formation of polykaryons. Thus, the hypotonic treatment presumably elicited permeability changes that caused the cells to swell (Pasternak, 1984). Moreover, the bridge interconnecting the cells was identified by electron microscopy as a virus particle and it was proposed to have fused with the two apposed plasma membranes (cf. Knutton and Pasternak, 1979). Whether this mechanism is crucial to virus-induced cell fusion will be discussed below but, evidently, fusion appears to be a local event, preceding the swelling that leads to microscopically detectable polykaryon formation. Further evidence supporting a local fusion event and, thus, the recognition of fusion and cell swelling as different steps in the overall process of virus-induced cell fusion, was provided by experiments showing that fluorescently labeled albumin, entrapped in one population of erythrocyte ghost, was transferred into a second population as a result of Sendai virus-induced fusion between ghosts under conditions that suppressed cell swelling (Sekiguchi *et al.*, 1981). Moreover, by employing a fluorescence assay that allows the continuous monitoring of fusion between ghosts, it has been shown that Sendai virus-induced cell fusion is initiated in less than 1 min, after exposing the aggregated virus–cell complex to 37°C (Hoekstra and Klappe, 1986b). Within this time interval a significant, let alone a massive, lateral redistribution of intramembrane particles is not observed (cf. Sekiguchi and Asano, 1978). Hence, such changes appear to occur as a consequence of fusion and presumably are the result of cell swelling.

There are several recent indications that the bridge mechanism, involving the simultaneous fusion of a virus particle with two apposed membranes, may not be the sole mechanism to explain virus-induced cell fusion; or rather, that virus–cell and cell–cell fusion should be seen as separate events (Fig. 3). Reconstituted Sendai virus envelopes (RSVE) fuse with erythrocytes or eukaryotic cells as efficiently as native virus particles (Harmsen *et al.*, 1985; Henis and Gutman, 1987). Yet, compared to the intact virus, their ability to induce cell fusion is much less. The structure of RSVE differs from the native virus in that the M protein is no longer present. Consequently, the enhanced rate of fusion for RSVE, relative to that of the native virus, has been explained by an enhanced mobility of the viral membrane proteins in RSVE. (As will be discussed below, mobility constraints are important parameters in virus fusion activity). By employing a technique that measures the lateral mobility of fluorescently labeled viral proteins, it was found that the *fraction* of these proteins that became laterally mobile in the erythrocyte membrane following fusion was markedly lower in the case of RSVE than in the case of intact virions (Aroeti and Henis, 1988). Interestingly, no relationship was found between the initial *rate* of virus or RSVE fusion and the *extent* of cell fusion. The secondary nature of cell fusion was further supported by the observation that after completion of virus–cell fusion at 25°C, where little cell fusion occurs, followed by an incubation at 37°C, cell–cell fusion increased to the level seen, when the entire incubation had been carried out at 37°C, i.e., conditions at which the relative fractional mobility is higher (Aroeti and Henis, 1988). The reason for part of the Sendai proteins of reconstituted envelopes to become less mobile in the plane of the plasma membrane, thus diminishing the efficiency of cell fusion, is unclear. Possibly, a rapid association with plasma membrane components may occur, given that the viral membrane glycoproteins are no longer attached to the matrix protein. However, it should also be taken into account that for cell fusion, occurring in cell suspensions, a tight virus-mediated intercellular interaction is necessary (Hoekstra and Klappe, 1986b). RSVE appear to cause a less tighter association between cells than intact viruses as RSVE readily dissociate at elevated temperatures after binding at low temperature (Klappe and Hoekstra, unpublished observation). Although further work is evidently needed, it is apparent that simultaneous fusion of a virus particle with adjacent cells is not needed to accomplish cell fusion. Studies carried out with virus-infected cells, resulting in FFWI under appropriate conditions, support this view.

Virus-Induced Fusion "From Within"

For revealing the details of molecular factors involved in virus fusion, studies dealing with fusion "from within" have been of greater significance than those dealing with fusion "from without." After infection and appearance

of the newly synthesized viral membrane proteins at the plasma membrane, and provided that they have been activated (if necessary) by proteolytic cleavage, large polykaryons are formed at neutral pH when the cells are infected with paramyxoviruses (Poste and Pasternak, 1978). For myxo-, toga-, and rhabdomembrane proteins a brief exposure to mild acidic pH is necessary (Nishiyama *et al.*, 1976; Chany-Fournier *et al.*, 1977). Fusion occurs rapidly (i.e., the reaction is triggered within seconds after lowering the pH and completed within several minutes), as monitored with kinetic assays. For polykaryons to be observed microscopically, a time interval of at least 20 min is required (Kempf *et al.*, 1987; Morris *et al.*, 1989). The conditions are analogous to those required for FFWO (White *et al.*, 1981) except that the induction of cell–cell fusion does not depend on an intact viral particle. Indeed, expression of the viral fusion protein alone suffices for triggering cell fusion. Furthermore, for FFWI to occur, infection and/or expression of viral membrane proteins in *both* apposed cell membranes is not necessary (cf. Fig. 3). When viral protein-expressing cells (HIV or mouse hepatitis virus) are co-cultured with uninfected cells, cell–cell fusion can be triggered (Dalgleish *et al.*, 1984; Daya *et al.*, 1988). This implies that viral protein-induced modifications in apposed cell surfaces are not required for cell–cell fusion and, indirectly, argues against a bridge-concept in FFWO as the sole mechanism for cell–cell fusion. In order to induce FFWI, the viral protein must be structurally intact, organized oligomerically, and properly exposed, i.e., anchorage in a membrane (the plasma membrane in this case) is necessary. Additionally, the cell culture should be confluent in order to allow for intercellular contact. Furthermore, the density of the expressed fusion protein is equally important (Fig. 3). For example, fusion between various HA expressing mammalian cells and erythrocytes was not observed when the density was less than approximately 10^6 HA molecules/cell (Sambrook *et al.*, 1985).

For many, if not all, virions that enter the cell by receptor-mediated endocytosis, binding and fusion reside in the same protein. For paramyxoviruses these functions are located on separate proteins, F (fusion) and HN (binding). Both proteins of Simian virus 5 have been expressed in CV-1 cells from cloned cDNAs (Paterson *et al.*, 1985). Syncytia formation was triggered at neutral pH, as anticipated, but expression of F alone sufficed for causing cell–cell fusion. This observation is quite distinct from FFWO. In the latter case HN is essential for viral binding and, generally, HN-devoid viral particles do not fuse. Moreover, it is thought that HN may play an active, though indirect, role in the fusion of the intact virus (Ozawa *et al.*, 1979; Miura *et al.*, 1982; Gitman and Loyter, 1984). Hence, FFWI may, in certain cases, not resemble in every respect the fusion of an intact virus particle *per se*.

It is obvious that FFWI is blocked when viral protein synthesis is inhibited (Koblet *et al.*, 1987). It is less clear how ATP affects virus-induced cell fusion (Kempf *et al.*, 1987; Ohnishi, 1988). FFWI of SFV-infected *Aedes albopictus* (mosquito) cells is blocked by ATP-depleting agents. Maintaining similarly infected BHK cells for a prolonged time interval at pH 6.0 inhibits FFWI, and a rapid depletion of ATP was noted. Concomitantly a change in membrane potential was observed due to a triggering of ion fluxes. Consequently, it was proposed that this change in membrane potential affected the fusion event (Kempf *et al.*, 1988). Recently, Morris *et al.* (1989) reported that influenza HA-mediated fusion between fibroblasts, expressing HA *c*DNA, and erythrocyte ghosts occurs irrespective of cell viability. This would indicate that neither metabolic energy nor membrane potential is required for cell fusion, consistent with the ability of viruses *per se* to fuse with artificial bilayers (see below). Furthermore, these results also indicate that osmotic gradients are not necessary, which is consistent with observations made on influenza virus-induced fusion of erythrocyte ghosts (Herrmann *et al.*, 1988).

Viral Envelope Proteins and Membrane Fusion

A variety of separate steps can be recognized, occurring prior to and during virus–cell fusion. Fundamental studies of the interaction between artificial membranes may serve, in this respect, as an illuminating model for dissecting these distinct steps and recognizing the forces that modulate each reaction (Rand, 1981; Israelachvili, 1985; Düzgüneş, 1985; Bentz and Ellens, 1988; Hoekstra and Wilschut, 1989). Thus, the overall event of virus–cell fusion can be roughly discerned in: (i) virus binding to the cell surface; (ii) interaction of the fusion protein with the target membrane; (iii) a perturbation of the intermembrane contact site; (iv) the actual merging event. These aspects will be discussed in the following sections.

Potential Forces Interfering with Virus–Target Membrane Interaction

Several barriers have to be overcome before fusion can be triggered. They include steric barriers, due to protruding cell surface proteins, and/or glycolipid carbohydrate head groups (Hoekstra and Düzgüneş, 1986, 1989). In addition, the presence of charges may give rise to electrostatic repulsions. For Sendai virus interacting with erythrocyte ghosts, the aggregation rate constant, reflecting the balance of forces that mediates viral binding, is close to the maximal value expected for a diffusion controlled process (Nir *et al.*, 1986a). This indicates that for viral binding to a biological target membrane,

these barriers have essentially no significance. This is in contrast to the interaction of Sendai virus with negatively charged phospholipid vesicles, in which case the aggregation rate constant is about an order of magnitude lower (Nir *et al.*, 1986b). The subsequent close approach of viral and target membrane, i.e., *after* viral binding, seems to sense a steric barrier, as the rate of virus–cell fusion (but also virus-induced cell fusion) is enhanced after trypsinization of the cell's surface (Lyles and Landsberger, 1979; Burness and Pardoe, 1981; Hoekstra and Klappe, 1986b).

In addition to steric barriers, yet another barrier, much more prominent, may emerge during the fusion-susceptible approach of virus and target membrane. This barrier consists of water, bound to cell surface molecules, including the lipid head groups at the bilayer/water interface. Since water stabilizes the bilayer structure and since both the viral and plasma membrane have to depart from the bilayer arrangement for fusion to occur, these stabilizing molecules will have to be removed. This scenario is derived from extensive work carried out with artificial membranes (for references, see Hoekstra and Wilschut, 1989). The hydration forces, i.e., the work required to remove water, is considered to represent the main barrier for fusion of artificial membranes. In these latter systems, hydration forces turn out to be the dominant repulsive forces at intermembrane distances less than 30 Å. It has been estimated that an external pressure of 10^3–10^4 atm is required to overcome the hydration barrier (Leikin *et al.*, 1987). Analogous to these observations in artificial systems, one tends to anticipate that these forces could be active during interaction of biological membranes as well (Rand and Parsegian, 1984). If so, it seems reasonable to assume that one of the properties of a viral fusion protein should be its ability to modify the contact interface by dehydration. Alternatively, one may then expect dehydration to affect viral fusion activity.

Effect of Viral Protein Mobility on Virus–Cell Fusion

To elucidate the parameters that modulate virus fusion, and thereby gain insight into the molecular details of virus fusion activity, a series of model systems have been used, including lipid vesicles, planar bilayers, and erythrocyte ghosts. A comparison of the different model systems has shown that certain fusion properties of the virions may reflect properties associated with the model system rather than with the virus (Klappe *et al.*, 1986; Stegmann *et al.*, 1986; Hoekstra and Kok, 1989). A note of caution with respect to direct extrapolation of such results as reflecting the *natural* fusion properties of a virus is therefore warranted.

Fusion of an envelope virus does not require extra viral stimuli, i.e., exogenous molecular factors such as divalent cations are not necessary for

the initiation of viral fusion. Major parameters that do affect fusion are the temperature and the pH.

With decreasing temperature the rate of virus fusion decreases. Optimal fusion activity is seen around 37°C, whereas below 20°C the virions display very little, if any, tendency to fuse (Marsh *et al.*, 1983; Lee *et al.*, 1983; Haywood and Boyer, 1984; Hoekstra *et al.*, 1985; Yamada and Ohnishi, 1986; Morris *et al.*, 1989). Diminished binding of the virus to the (cellular) membrane or changes in membrane fluidity can be excluded as the cause of temperature-dependent inhibition. Rather, the work of Cherry and coworkers (Lee *et al.*, 1983; Junankar and Cherry, 1986) has revealed that motional constraints of the viral membrane proteins drastically affect the ability of a virus to fuse. By measuring the decay of flash-induced transient dichroism of eosin triplet probes, attached to the viral proteins, they observed that the rotational mobility of the proteins was very low below 20°C. Furthermore, by labeling the membrane proteins of Sendai virus (HN and F) with the fluorophore eosinyl-5-maleimide and measuring the fluorescence as a function of temperature, substantial self-quenching of the fluorescence is seen at temperatures where fusion does not occur (Hoekstra *et al.*, 1989). Around 22°C, when fusion between Sendai virus and erythrocyte membranes dramatically increases, a rather abrupt relief of fluorescence self-quenching occurs. Taken together, the results suggest that at lower temperatures the viral membrane proteins display a tendency to cluster, which imposes mobility constraints that have to be relieved in order to engage in the process of membrane fusion. Thus, motional freedom of the fusion protein is essential for virus fusion activity. In this respect a "natural" tendency of viral membrane proteins to cluster has been observed before by electron microscopy (Kim *et al.*, 1979), an effect that might be controlled, among others, by transmembrane interactions between the spikes and the matrix protein. This control and its consequences for virus fusion may also be inferred from observations that cell–cell fusion is substantially more efficient when induced by intact Sendai virus than by its reconstituted envelopes, lacking the matrix protein (Henis and Gutman, 1987). A limited fractional mobility may not significantly hamper virus–cell fusion, but when assuming that virus–cell and virus-induced cell fusion can be considered as separate events and that the latter depends on the density of *mobile* fusion proteins (which is less in the case of reconstituted envelopes) in the plane of the plasma membrane (cf. Sambrook *et al.*, 1985), it follows that cell–cell fusion may become less efficient.

Role of Viral Protein Conformation

Paramyxoviruses fuse with plasma membranes at neutral pH (Choppin *et al.*, 1981; Hoekstra and Kok, 1989). At mild acidic or basic pH an

irreversible conformational change is triggered in the F protein, resulting in a strongly reduced, but not completely abolished, fusion of the virus with erythrocytes (Hsu *et al.*, 1982; Hoekstra *et al.*, 1985).

The requirement of a mild acidic pH for those virions that enter the cell by receptor-mediated endocytosis has been best characterized in the case of fusion of influenza virus. The fusion of influenza is highly cooperative and requires a critical number of acid-activated trimers (Doms *et al.*, 1985; Doms and Helenius, 1988), which presumably allows the HA trimer to undergo the irreversible conformational change in a rapid, concerted manner. Mixed trimers, i.e., hybrids formed from distinct HA monomeric subunits each displaying a defined pH threshold for the conformational change, display fusion activity at a pH intermediate to that of the monomeric subunits (as homotrimers), which coincides with the conformational change and exposure of the previously concealed hydrophobic N-terminal region in HA_2 (Boulay *et al.*, 1988).

The timing of the acid-induced conformational change is important. In the absence of a target membrane, viral fusion activity is almost instantaneously inactivated when the virus is incubated above 20°C. Both hemolysis and fusion, as monitored with a fluorescence assay based on lipid mixing, are abolished, when acid-pretrated virus is incubated with erythrocytes and ghosts, respectively (Junankar and Cherry, 1987; Stegmann *et al.*, 1986). Under these conditions the viral proteins interact with each other rather than with the target membrane, presumably due to hydrophobic interactions between exposed HA_2 subunits (Doms *et al.*, 1985; Ruigrok *et al.*, 1986a, b; Junankar and Cherry, 1986). Interestingly, when the acid activation is carried out at temperatures below 20°C the fusion activity is not substantially inactivated. This protective effect is likely to be attributed to a restricted lateral and rotational mobility of the proteins at low temperature, which apparently prevents intermolecular hydrophobic interactions (see above; Junankar and Cherry, 1986). On the other hand, it cannot entirely be excluded that the spike is only partly unfolded at lower temperatures. In this respect, it should also be noted that the "conformational change," referred to in essentially all studies cited, reflects the conformation determined at *neutral* pH, i.e., the protein is neutralized after acidification.

The ectodomains of both E_1 and E_2 of SFV undergo an irreversible conformational change when exposed to acidic pH (Kielian and Helenius, 1985). The conformational change coincides with the threshold pH for virus fusion (Kielian and Helenius, 1985). *wt*SFV displays a pH threshold of 6.2 for both events. However, a pH of 5.3 is required in the case of the SFV mutant *fus*-1. In passing, it is interesting to note that, when probing the conformational change in E_1 with specific mAbs during interaction of both viruses with CHO and BHK cells, a half time of ca. 5 min was seen for

conversion of *wt*E_1 to the acidic form, whereas *fus*-1 E_1 showed a half time of ca. 15 min (Kielian and Sayad, 1990). Taken together, these observations could imply that the intracellular sites of penetration for both viruses may differ (early vs. late endosomes).

E_1 becomes trypsin-resistant, whereas E_2 becomes trypsin sensitive, at low pH. Intermolecular interaction between E_1 and E_2 is not required for the change in conformation to occur. The reason as to why E_2 should undergo a conformational change is unclear and might not be related to the virus ability to engage in fusion. Recent work of Omar and Koblet (1988) suggests that SFV, containing E_1 only, is infectious and causes the fusion of mosquito cells. E_1-containing virus was prepared by taking advantage of the possibility of completely digesting E_2 (and E_3) with trypsin after a low pH treatment of the virus. Indirectly, these results also indicate that SFV is more resistant toward acidic pH inactivation that influenza virus. This would be consistent with observations that detergent binding (to previously concealed hydrophobic sites) does not drastically increase after acidic exposure (Kielian and Helenius, 1985).

The G protein of VSV, although requiring mild acidic pH to become fusogenic, differs from E_1 and HA in that the conformational change appears to be reversible (Crimmins *et al.*, 1983). In fact, a preincubation of the virus alone at low pH stimulates the fusion reaction, as revealed by direct monitoring of the merging of the virus with cultured cells (Puri *et al.*, 1988).

From the foregoing it is clear that although influenza virus, SFV, and VSV share a common pathway of entry, several subtle differences exist as to the molecular properties of their fusion proteins. It has been noted before that their rates of entry may also differ (Marsh, 1984).

Relevance of Hydrophobic Interactions in Virus–Cell Fusion

Once bound to a target membrane, influenza HA may rapidly engage in hydrophobic interactions, when activated by mild acidic pH. This can be inferred from observations showing a rapid inactivation of the virus when activated in the absence of a target membrane. Experimental data suggest that the irreversible pH-activated conformational change of HA (Japan strain) may be completed within ca. 15 sec (Morris *et al.*, 1989). A similar time interval of activation has been reported for E_1 and E_2 of SFV (Kielian and Helenius, 1985). The activation time of HA was derived from results obtained when studying the fusion of prebound erythrocyte ghosts with HA-expressing fibroblasts (Morris *et al.*, 1989). Fusion was monitored by labeling the ghost with a self-quenching concentration of a fluorescent lipid analogue and following the relief of quenching when the ghosts fused with the

fibroblasts. When the system, at 37°C, was acid activated, a lag phase of 30 sec was apparent, prior to initiation of lipid dilution. A similar lag phase is commonly seen when Sendai virus fuses with erythrocytes (Hoekstra *et al.*, 1985). However, when influenza virus *per se* fuses with erythrocytes, no significant lag phase can be detected (Stegmann *et al.*, 1986); neither was a lag phase between acid activation and the initiation of fusion apparent when in analogous experiments fusion was studied between viral receptor-containing liposomes with HA-expressing cells (Van Meer *et al.*, 1985). This makes it uncertain as to whether the lag phase observed by Morris *et al.* (1989) was due to a specific property of HA or to the experimental system. It was suggested that the lag phase might be related to the time required for mobilization of activated HA at the site of fusion. However, this seems rather unlikely, given the fast activation time (< 15 sec), which would rather result in a rapid inactivation due to intermolecular hydrophobic interactions, as discussed above. Furthermore, if mobilization to "fusion sites" were possible, one would anticipate that FFWI would be largely independent of viral protein density, which is evidently not the case. Nevertheless, it is clear that in this system HA engages in hydrophobic interactions before any fusion is apparent. This can be inferred from the observation that pH changes of the medium carried out during the lag phase are reflected in the fusion kinetics, initiated after 30 sec. A brief activation of less than 15 secs (i.e., the time required to completely activate HA) followed by neutralization shows a diminished fraction of fusion, suggesting that only a fraction of HA is involved or that there is a partial involvement of all molecules. However, consistent with various other studies (Stegmann *et al.*, 1986), fusion appears to be modifiable by pH manipulation, and a low pH is necessary to allow the fusion reaction to proceed, which is presumably related to the need to protonate acid amino acid residues, contained in the fusogenic HA_2 peptide (see below).

Studies dealing with the interaction of erythrocyte ghosts or liposomes with HA-expressing cells (Doxsey *et al.*, 1985; Van Meer *et al.*, 1985; Morris *et al.*, 1989) reveal that two kinetically distinct steps can be discerned in the overall fusion process: (i) a step which changes the mode of the interacting membranes from electrostatic to, presumably, hydrophobic (as it requires proteolytic cleavage of HA), and (ii) the fusion reaction itself. In both systems the first step is revealed by effects of fusion "reversibility" by readjustment of the pH (Morris *et al.*, 1989) and by neuraminidase resistance of ghosts or liposomes, which have not yet fused with the cells (Doxsey *et al.*, 1985; Van Meer *et al.*, 1985). Note that neuraminidase cleaves sialic acid and may therefore destroy the binding between viral protein and its receptor as long as the interaction is purely electrostatic. From these studies one may also infer that the changes in binding mode already can occur at temperatures

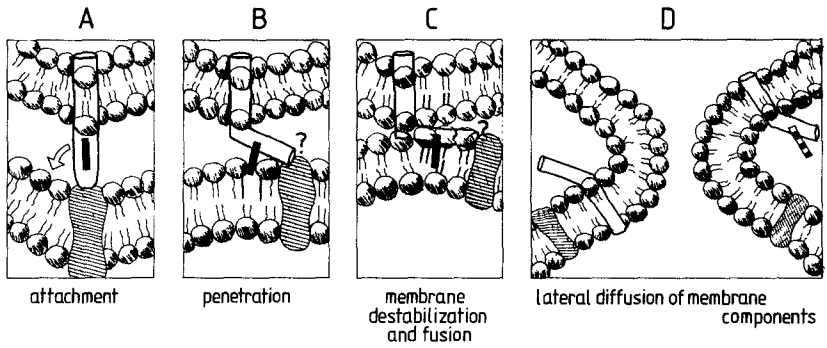

Fig. 4. Hypothetical stages in the mechanism of fusion of enveloped viruses. (A) Attachment of the virus (top) to a target membrane receptor. For paramyxoviruses binding is mediated by a separate protein (HN). A second protein, F, triggers the fusion reaction. For most other viruses fusing in the endosomal compartment, binding and fusion activity reside in the same protein, albeit at different sites. (B) At neutral pH (paramyxoviruses) or after exposure to mild-acidic pH (myxo-, toga-, and rhabdoviruses), the exposed or previously concealed (cf. Fig. 2), hydrophobic amino acid domain penetrates into the target membrane. Note that the intermembrane distance may still be substantial, which makes it likely that additional changes in protein structure have to take place. The latter, possibly triggered by the hydrophobic dehydration as initiated in (B), may further facilitate the close approach of viral and target membrane, necessary for establishing fusion-susceptible interbilayer contact. Consequently, interfacial water is expelled (C), causing the membranes to destabilize locally such that fusion takes place. Subsequently, randomization of the viral and cellular membrane components takes place (D).

below 37°C, implying that a conformational change may take place (at least partially) independent of temperature (Kielian and Helenius, 1985; Doms *et al.*, 1985). An important question that remains to be answered is whether (in terms of hydrophobic interactions) HA_2 interacts solely with the target membrane or that other parts of the HA molecule are involved as well. That the hydrophobic segment of HA_2 and, as far as determined, similar segments present in other fusogenic viral proteins trigger fusion by direct, hydrophobic penetration into the target membrane has been a longstanding hypothesis (Fig. 4).

Direct evidence for penetration of hydrophobic segments, contained in viral fusion proteins, into the target membrane is gradually emerging. By employing carbene-generating photoaffinity probes, located in the hydrophobic core of a liposomal bilayer, it has been shown that the interaction of bromelain-cleaved HA with such bilayers is mediated, after acid activation, by the BHA_2 subunit (Harter *et al.*, 1988). A detailed analysis of the distribution of the photolabel within the BHA_2 subunit showed that the NH_2-terminal of 21 amino acids is responsible for this interaction and that this segment probably adopts a helical conformation when inserted into the membrane (Harter *et al.*, 1989). Although these experiments were carried out with nonfusogenic, cleaved HA, while penetration was established under

equilibrium conditions, these results are entirely consistent with the current hypothesis of viral fusion (Fig. 4).

In order to obtain direct mechanistic information about the role of hydrophobic interactions between a viral fusion peptide and a membrane involved in fusion, it would be desirable to carry out photoactivation *during* the initial stages of the fusion reaction. This type of time-dependent photolabeling was recently reported during fusion between Sendai virus and liposomes (Novick and Hoekstra, 1988). Consistent with the proposed hydrophobic insertion of F_1 into a target membrane, it was found that ca. 80% of the photolabel became associated with this protein during the early interaction stages. Similar results were found when such experiments were carried out with erythrocytes as target membrane (Novick and Hoekstra, unpublished). Penetration appears to occur faster than the kinetics of fusion, assayed by lipid mixing, implying an overall fusion mechanism consisting of more than one step (see below).

Hydrophobic interactions of the viral membrane proteins do not seem to be limited to the process of penetration into the hydrophobic core of the target membrane, as discussed above. The potential significance of hydrophobic interactions in virus–target membrane interaction prior to and, in particular, after the penetration step has also been suggested for SFV, as revealed by hydrophobic interaction chromatography (Omar and Koblet, 1988). It was observed that the virus binds to such a column at neutral pH but competition with a nonionic detergent caused the particle to dissociate rapidly. A mild-acid pH activation of the virus, *prior to* binding, still resulted in dissociation of most of the viruses. However, when the virus was activated *after* binding at neutral pH, almost all virions remained firmly associated with the column. Thus, in a hydrophobic environment, the effect of mild acidic pH potentiates viral hydrophobicity (Omar and Koblet, 1988). These results could imply that proper viral protein unfolding, triggered by acidic pH, not only requires a proper binding—prior to unfolding—but also may involve subsequent structural changes before the viral membranes can participate in fusion.

In this regard it should also be taken into account that after penetration of the hydrophobic fusion peptides the interbilayer distance between viral and target membrane can still be on the order of several tenths of angstroms, given that the hydrophobic sequence of HA_2 is located about 30 Å from the viral interface (Wiley and Skehel, 1987), while in the case of Sendai F_1 it might be up to ca. 80 Å (cf. Hoekstra and Kok, 1989). Hence, it seems not unreasonable to assume that additional structural changes are needed that might be hydrophobic in nature, given the strong repulsive hydration forces that occur during the process of close approach. It is possible that such changes are triggered as a result of the hydrophobic dehydration caused by

penetration of the hydrophobic peptide into a target membrane (cf. Hoekstra and Wilschut, 1989; Hoekstra *et al.*, 1989). Of interest in this respect are observations that the initial fusion rate of Sendai virus with erythrocyte ghosts is dramatically increased when the fusion medium contains a relatively small amount (4 wt. %) of the dehydrating agent polyethylene glycol (PEG; Hoekstra *et al.*, 1989). Moreover, a preincubation of the virus *per se*—but not erythrocyte ghosts—in PEG causes an additional doubling of the initial fusion rate. The stimulating effect was due to the fusion reaction itself, as the fusion rate constant increased in the presence of PEG by almost an order of magnitude, while the aggregation rate constant increased marginally. These results may imply that a hydrophobic environment, mimicked by the presence of PEG, facilitates the conformational change(s) needed to bring the viral and target membrane into direct contact. Under physiological conditions these changes may be brought about as a result of hydrophobic penetration, as outlined above. The delay between penetration—as revealed by photoaffinity labeling—and the onset of fusion—monitored by a fluorescence assay—during interaction between Sendai virus and erythrocytes (Novick and Hoekstra, unpublished observations, see above) may reflect the time required for such structural changes to occur.

To summarize the foregoing discussion, it appears possible that regions other than the hydrophobic fusion sequences are equally relevant for the overall fusogenic properties of a viral protein. In fact, in several fusion proteins the presence of additional hydrophobic stretches has been noted (e.g. Blumberg *et al.*, 1985; Schlesinger and Schlesinger, 1986). To further characterize functionally important regions in viral proteins, including those referred to above, site-specific amino acid mutations and/or the creation of new N-glycosylation sites could be powerful tools in this respect (Gething *et al.*, 1986a; Gallagher *et al.*, 1988).

Probing of Functional Regions in Viral Fusion Proteins

Seemingly subtle substitutions of amino acids (cf. Beyer *et al.*, 1986) can have relatively drastic effects on the threshold pH for viral fusion (Doms *et al.*, 1986), which is closely related to the pH at which the conformational change is triggered. It is thought that the distinctions in pH threshold between strains and subtype are due to the relative displacement of structural domains (e.g., elimination of electrostatic and hydrophobic interactions) occurring during the conformational change, rather than to a direct protonation of single amino acids (Wharton *et al.*, 1988). Apart from "spontaneous" substitutions, specific mutations can be introduced by oligonucleotide-directed mutagenesis. Such an approach has been used by Gething and coworkers

(1986b), and their work confirmed the central role played by the fusion peptide in HA_2. When substituting a Glu residue for Gly at position 1 (see Table II), fusion is completely abolished as determined by assays based on cell–cell fusion between HA-expressing cells (FFWI) and fusion of erythrocyte ghosts with the HA-expressing cells. In this case, a conformational change took place, as revealed by proteinase K digestion, albeit less rapid and to a lower extent than that observed in *wt*HA. When Glu was introduced for Gly at position 4 the pH threshold for both types of fusion was raised and the fusion efficiency was lowered compared to *wt*. Yet, the rate of the conformational change was faster than for *wt*. A remarkable phenomenon was seen when the Glu residue at position 11 was substituted for Gly. In this case fusion between erythrocytes and cells was normal (i.e., the same as *wt*HA) but polykaron formation was greatly impaired, in spite of the fact that the pH threshold and the kinetics of the conformational change were essentially similar to those observed for *wt*HA. No explanation was presented for these observations. It is possible, however, that incomplete protonation of Glu at the N-terminus might explain the abolishment of fusion. It is also possible that relatively subtle changes in the conformation—having serious consequences for fusion—went unnoticed by proteinase K digestion. However, why elongation of the hydrophobic stretch (Gly for Glu at position 11) impaired cell–cell fusion but *not* erythrocyte ghost–cell fusion, remains an enigma, unless the encapsulated fusion-reporting marker in the ghosts could have obtained access to the cells by a viral spike-mediated perturbation of apposed membranes, rather than by fusion.

Addition of carbohydrates at novel positions on nascent polypeptide chains (after oligonucleotide-directed mutagenesis) provides another promising tool for probing functional (and structural) amino acid regions in viral fusion proteins (Gallagher *et al.*, 1988). Thus, glycosylation of novel sites may result in shielding or disruption of functional epitopes on the surface of viral proteins. Viral membrane proteins are glycosylated, like many membrane and secretory proteins, but the functional role of glycosylation is still unclear. It has been shown for some, but not all, glycoproteins that glycosylation stabilizes their structure and plays a role in intracellular transport and trafficking (Elbein, 1987; Hoekstra and Düzgüneş, 1989). When glycosylation is inhibited in virally infected cultured cells, the maturation and release of several enveloped virions are prevented (Gandhi *et al.*, 1972; Kaluza *et al.*, 1972). On the other hand, correct glycosylation of SFV membrane proteins appears not to be a prerequisite for E_1-induced fusion "from within" of a mosquito cell line (Naim and Koblet, 1988) although virus-budding was found to be inhibited.

Role of Target Membrane: Effect of Cholesterol on Virus Fusion

Little attention has been payed thus far to whether a virus fuses randomly at the cell surface or that specific molecular requirements, at the level of the target membrane, have to be met for fusion. Relevant to such a question is also the specificity of viral receptors. Many virions display binding specificity toward sialic acid, abundantly present at the cell surface, and a range of lipids and proteins containing this charged sugar residue can act as viral receptor (for references, see Hoekstra and Düzgüneş, 1989). Unambiguous proof for high receptor specificity has only been shown for HIV, which via gp120 specifically binds to CD4 (Sattentau and Weiss, 1988). The structure of this receptor has been well characterized, including the binding domain for HIV (Chao *et al.*, 1989). Interestingly, transfection of mouse cells with cloned human CD4 *c*DNA resulted in binding of the virus, but not fusion (Maddon *et al.*, 1986) whereas human CD_4^- cells became permissive for fusion. One possibility that might explain this result is that the molecular environment of the receptor codetermines the ability of a virus to penetrate.

In particular the use of liposomes has suggested that various physical parameters, such as bilayer packing and state of surface hydration, may affect the ability of a virus to fuse (Klappe *et al.*, 1986; Nir *et al.*, 1986b). Such studies have also revealed that some viruses require the presence of cholesterol in order to fuse. However, the role of cholesterol in viral fusion is controversial. For example, Haywood and Boyer (1984) observed no effect of cholesterol on Sendai virus interacting with liposomes consisting of zwitterionic phospholipids and gangliosides as viral receptors. On the other hand, Kundrot *et al.* (1983) demonstrated a requirement for cholesterol in Sendai virus-induced lysis of liposomes. Cholesterol does not seem to affect influenza virus fusion (Maeda *et al.*, 1981; White *et al.*, 1982b) but is indispensable for SFV fusion activity (White and Helenius, 1980; Kielian and Helenius, 1984, 1985).

In trying to analyze how cholesterol may modulate virus fusion, at least two aspects should be taken into account: (i) cholesterol may modify the bilayer properties, including the lateral distribution of proteins, when supplemented to cholesterol-poor membranes, and/or (ii) specific interactions between viral proteins and cholestrol may be involved.

Cholesterol is known to regulate membrane fluidity (i.e., it fluidizes solid membranes and solidifies fluid membranes) and to decrease membrane permeability (Yeagle, 1988). Cholesterol may also modify repulsions between adjacent membranes by interfering with the organization of interfacial water as it decreases the depth to which water penetrates into bilayers (Simon *et al.*, 1982). Steric repulsions between apposing membranes can be reduced by

cholesterol as a result of its ability to spread lipid molecules apart in the bilayer, i.e., cholesterol can effectively reduce the density of phospholipid head groups in the plane of the bilayer (McIntosh *et al.*, 1989). As a result, the "accessibility" of the bilayer surface may become enhanced in processes involving squeezing of membranes, as occurs during fusion.

Some recent work, in which fusion of Sendai and influenza virus with bacterial membranes (Citovsky *et al.*, 1988) and mouse hepatitis virus (MHV)-induced cell fusion were studied (Daya *et al.*, 1988), has demonstrated pronounced effects of cholesterol in promoting virus fusion. In both studies it was observed that little fusion (virus–cell fusion, monitored by a fluorescence assay and microscopy, and cell–cell fusion, determined by microscopy, respectively) occurred with or of cholesterol-poor cells. Supplementation of cholesterol greatly facilitated fusion, and merging increased with increasing cholesterol concentration. In the former study no kinetic data were presented and it is not clear therefore whether only the rate of fusion increased and/or whether the final extent of fusion also increased. MHV-induced cell fusion showed a more rapid kinetics as a result of cholesterol supplementation. Internalization of the virus was not enhanced, but no data were provided as to whether the kinetics of virus–endosome fusion increased as well. Virus replication, in terms of virus production, was not enhanced, but it cannot be excluded that cholesterol supplementation interfered with virus budding. Interestingly, cell fusion also increased when MHV virus-infected cells were incubated with uninfected, cholesterol-supplemented cells, indicating that cholesterol in the *target membrane* modulated the fusion in particular, consistent with the studies in which fusion of influenza and Sendai virus with bacterial cells was examined directly. For Semliki Forest virus, evidence has been provided that suggests that in order for E_1 to undergo a proper acid-induced conformational change, cholesterol is necessary (Kielian and Helenius, 1985). This conformational change, probed by conformation-specific antibodies (Kielian and Sayad, 1990), can proceed normally in the intact virus, as also reflected by a resistance of E_1 to trypsinization at acidic pH. The solubilized ectodomain of E_1 requires cholesterol, incorporated into a liposomal membrane, for mimicking the properties of the intact E_1 molecule. Since SFV does not fuse with cholesterol-free membranes, it seems likely that the virus needs cholesterol in the target membrane to fuse. However, a tight association between E_1 and cholesterol has not been demonstrated thus far. For the F_1 protein of Sendai virus, such as interaction has been suggested and, moreover, it has been claimed that the fusogenic sequence of F_1 (N-terminus) was directly involved in this association by showing that small peptides, interacting with the same sequence, could compete with cholesterol (Asano and Asano, 1988). Remarkably, the interaction was not dependent on the 3-β-hydroxyl "head group" of the cholesterol, as seen for

SFV E_1. It is therefore difficult to comprehend the specificity of F_1-cholesterol interaction, other than involving hydrophobic interactions.

A priori, a specific interaction of a protein with cholesterol would not be unprecedented. Also, certain polyene antibiotics, a group of naturally occurring pore-forming compounds, require an interaction with cholesterol to induce porelike structures (De Kruijff and Demel, 1974). Yet, the interaction of Sendai virus with cholesterol-supplemented bacterial cells did not induce leakage (Citovsky *et al.*, 1988). Furthermore, in systems involving fusion of liposomes, cholesterol appears to inhibit, rather than stimulate, fusion in many cases (Shavnin *et al.*, 1988). On the other hand, it has also been reported that the effect of cholesterol on Ca^{2+}-induced fusion of lipid vesicles may be critically controlled by the phospholipid composition of the membranes (Stamatos and Silvius, 1987). This has also been noted in a study in which the fusion between Sendai virus and liposomes, containing various mixtures of phospholipid and cholesterol, was examined (Tsao and Huang, 1985). It is possible, therefore, that the modulating effect of cholesterol on virus fusion stems, at least in part, from indirect effects.

Synthetic Peptides: Competition with and Modelling of Virus Fusion

To further probe the properties and functions of viral fusion proteins, synthetic peptides have been synthesized that resemble or mimic the N-terminal regions of the fusogenic polypeptides. One of the early applications of these peptides was to investigate whether they could compete with virus fusion (Richardson *et al.*, 1980; Richardson and Choppin, 1983). It was found that the peptide Z-D-Phe-L-Phe-Gly (in which Z is a carbobenzoxy group), matching the N-terminus of Sendai F_1, could inhibit viral infectivity, hemolysis, and cell–cell fusion "from within," without blocking virus adsorption to the cell surface. Interestingly, the inhibition was accomplished by an interaction of the peptide with the cell and not with the virus. From these experiments it was suggested that the peptide competed with F_1 for specific interaction sites on the target membrane. However, the *specificity* is questionable since for optimal inhibition both the carbobenzoxy group and the D-form of Phe in position 1 were necessary. Recently, evidence has been presented that such peptides may also directly interact with the N-terminal region of F_1 (Asano and Asano, 1988). In spite of these uncertainties in specificity other work has suggested that it is not unlikely that domains may exist on the cell surface for viral penetration. It has been found, for example, that the number of Sendai virus particles that fuse per cell (approximately 200 for erythrocytes) is much lower than the number of (fusion-active) virus particles that bind (approximately 1200). This indicates that fusion does not

take place "at random," sooner or later after a virus particle binds (cf. Hoekstra and Klappe, 1986a; Nir *et al.*, 1986a).

Thus far, synthetic peptides have been more profitably used in studies in which their interaction with liposomes or cells was examined. Such experiments have been carried out with peptides resembling the N-terminal regions of the G protein (VSV) and HA_2 (influenza). Although there is no evidence that supports a direct role of the N terminus of G in fusion of VSV, the region appears to be relatively conserved in several strains (see above). This domain is not particularly hydrophobic (for details, cf. Hoekstra and Kok, 1989 and references therein). Yet a peptide of six amino acids, corresponding to the N-terminal segment of G, caused hemolysis at pH 5.0 and, moreover, appeared to be ten times more potent in doing so than a peptide resembling the 25 amino acid terminal (Schlegel and Wade, 1985). However, only the latter peptide mimics closely the low pH dependence of hemolysis and permeabilizes cultured cells in a pH-dependent manner, similarly as observed for the native virus. The peptide did not induce cell–cell fusion. The fairly aspecific effect of the six amino acid peptide is due likely to its inability to adopt a defined structure because of its limited length (cf. Cantor and Schimmel, 1980). This can also be inferred from analogous work carried out with peptides resembling the N-terminus of HA_2. In this work (Lear and DeGrado, 1987) the interaction of N-terminal peptides, consisting of 16 (H_{16}) and 20 (H_{20}) amino acids with 1-palmitoyl-2 oleyl PC vesicles, was examined. The H_{20} bound in a helical conformation while H_{16} did not. Only H_{20} induced fusion, suggesting that helix formation is required for fusion. In this context a helical insertion of BHA_2 into membranes has also been proposed (Harter *et al.*, 1989). With the same peptide, however, fusion took place both at pH 5.0 and 7.0, which is evidently not seen for influenza virus itself.

Recently, Murata *et al.* (1987a) carried out very similar experiments using egg-PC vesicles and a synthetic peptide resembling the N-terminal of HA_2 of a strain different from that used by Lear and DeGrado. In this case peptide-induced fusion exactly matched the low pH characteristics of fusion of native viruses. The fusion was triggered around pH 6.0 and was abolished when the pH was brought back to neutral. The same authors (Murata *et al.*, 1987b) also carried out similar fusion experiments with a synthetic peptide resembling the natural hydrophobic peptide mellitin. Mellitin has been used before as a "model"-protein for protein-induced fusion of vesicles (cf. Hoekstra and Wilschut, 1989). The 26 amino acid peptide contains five basic amino acids, four of which are located at the carboxyl end. When added to egg-PC vesicles, fusion is induced both at acidic and neutral pH. When the positively charged groups were converted to carboxyl groups by succinylation, the peptide behaved as the viral fusion peptide: fusion only occurred at mild acidic pH and could be interrupted by returning the pH back to neutral.

It is clear from these studies that synthetic peptides can only partly mimic the complexity of the fusogenic properties of a viral protein. It is difficult to assess which factors in terms of peptide–bilayer interaction actually contribute to bringing about fusion. Evidently, the peptides mediate both functions of binding (aggregation) and fusion, normally carried out by distinct regions in the viral protein. Thus some of the peptides may align along the vesicle surface, while others will presumably penetrate, perhaps only partially. That a seemingly quite distinct peptide (mellitin) can minimic a viral peptide might suggest that the sequence of a viral fusion peptide is not a very specific factor, provided that it is hydrophobic and contains a few acidic, protonatable amino acids. However, the HA_2 N-terminal region forms a structurally-linked entity of the entire protein determined by inter-subunit interactions. These interactions appear to ultimately regulate as to when and where fusion is triggered, as governed by a mild acid pH-induced conformational change. One may conclude, therefore, that both structural and molecular factors are closely related and highly important parameters that govern viral fusion activity.

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