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Topical Review

Peptides and Membrane Fusion: Towards an Understanding of the Molecular Mechanism of Protein-Induced Fusion

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Abstract. Processes such as endo- or exocytosis, membrane recycling, fertilization and enveloped viruses infection require one or more critical membrane fusion reactions. A key feature in viral and cellular fusion phenomena is the involvement of specific fusion proteins. Among the few well-characterized fusion proteins are viral spike glycoproteins responsible for penetration of enveloped viruses into their host cells, and sperm proteins involved in sperm-egg fusion. In their sequences, these proteins possess a ''fusion peptide,'' a short segment (up to 20 amino acids) of relatively hydrophobic residues, commonly found in a membrane-anchored polypeptide chain. To simulate protein-mediated fusion, many studies on peptide-induced membrane fusion have been conducted on model membranes such as liposomes and have employed synthetic peptides corresponding to the putative fusion sequences of viral proteins, or *de novo* synthesized peptides. Here, the application of peptides as a model system to understand the molecular details of membrane fusion will be discussed in detail. Data obtained from these studies will be correlated to biological studies, in particular those that involve viral and sperm-egg systems. Structure-function relationships will be revealed, particularly in the context of protein-induced membrane perturbations and bilayer-to-nonbilayer transition underlying the mechanism of fusion. We will also focus on the involvement of lipid composition of membranes as a potential regulating factor of the topological fusion site in biological systems.

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Key words: Fusion protein — Peptide-induced fusion — Secondary structure — Membrane anchorage — Oligomerization — Molecular shape

Introduction

Membrane fusion reactions occur continuously in all eukaryotic cells, and are involved in processes such as endocytosis, intracellular transport and recycling of membrane components. Neurotransmission, fertilization or formation of myotubes also require the recognition and merging of formerly separate membranes. The entry of enveloped viruses into their host cells leading to infection is also accomplished by fusion between viral and cellular plasma or endosomal membranes. Fusion is an energetically unfavorable event since biological membranes are submitted to strong repulsive hydration, electrostatic and steric barriers [26, 81, 94]. These barriers can be overcome by membrane proteins, which facilitate local dehydration [209] and are thought to induce local perturbations in the lipid bilayer through their insertion into membranes [25, 97, 130]. Virus-cell fusion is the only biological membrane fusion event in which the proteins directly responsible for membrane merging have been identified unequivocally [64, 82, 84, 193]. Extensive work with this system has led to the general proposal that membrane fusion proteins would share common motifs (‘fusion peptide’), in particular a stretch of hydrophobic residues susceptible to interact with and destabilize a lipid bilayer. In recent years, candidate fusion proteins have been identified, which are involved in gamete fusion [3, 7, 134, 167, 183, 200, 207], in myoblast fusion [201], in vesicular fusion in neurons [169] or in

Table 1. Some viral and cellular glycoproteins

Virus	Fusion complex	Processing	Binding	Fusion peptide	References
Influenza virus	HA1/HA2	Yes	Yes	N-term. HA2	[17, 197]
Semliki Forest virus (SFV)	E1/E2/E3	Yes	Yes	Internal E1	[101, 161]
Tick-borne encephalitis virus (TBE)	E	No	Yes	Internal	[75, 152]
Rabies virus	G	No	Yes	Internal	[64]
Vesicular stomatitis virus (VSV)	G	No	Yes	Internal	[57, 64, 107]
Lymphocytic chorio-meningitis virus (LCMV)	G1/G2	Yes	Yes	Internal G2	[39]; Glushakova et al., 1992[a]
Human immunodeficiency virus (HIV)	gp120/gp41	Yes	Yes	N-term. gp41	[62]
Simian immunodeficiency virus (SIV)	gp120/gp32	Yes	Yes	N-term. gp32	[10]
Rous sarcoma virus (RSV)	gp85/gp37	Yes	Yes	Internal gp37	[91]
Murine leukemia virus (MLV)	gp70/p15E	Yes	Yes	N-term. p15E	[96]
Bovine leukemia virus (BLV)	gp51/gp30	Yes	Yes	N-term. gp30	[185]
Sendai virus and other paramyxoviruses	F1/F2 and HN	Yes	Yes (through HN)	N-term. F1	[108, 164]
Hepatitis B virus (HBV)	S	?	No	N-terminal	[155, 156]
Cell					
Sperm	Fertilin α and β	Yes	Yes	Internal α	[7, 167, 199, 200]
Myoblast	Meltrin α	Yes	Yes	Internal	[82, 200, 201]

[a] Glushakova, S.E. et al., 1992. *Biochim. Biophys. Acta* **1110**:202.

endocrine cells [2], in fusion events leading to nuclear pore complex assembly [70, 74] and to photoreceptor rod cell outer segments formation [9]. For some of these proteins, evidence concerning the presence of a fusion peptide has been obtained [7, 74, 82, 122, 132, 200]. With respect to insight into the molecular mechanism underlying the overall fusion event, large voids in such knowledge are still apparent. To understand how proteins modulate membrane fusion, we will integrate current structural and molecular insight that is of relevance to the mechanism of protein-induced membrane fusion, as obtained from studies with both natural and model fusogenic peptides and proteins.

The reader is referred to recent reviews specifically devoted to certain topics [5, 38, 41, 66, 113, 129, 134, 157, 158, 167, 200]. Careful attention will also be paid to the relevance of using synthetic peptides resembling the putative sequences of fusion peptides, as models for protein-induced fusion. Ultimately, we will attempt to answer the question: what can we learn from model peptide-induced fusion in order to explain biological fusion events induced by proteins?

Structural Features of Fusion Proteins: Similarities and Differences

Fusion of Viruses

To date, spike glycoproteins of enveloped viruses are by far the most studied and best characterized fusion proteins (Table 1). In spite of dissimilarities between viruses (e.g., genomic type, host range, entry pathway into host cells), viral fusion glycoproteins share several common features: they protrude *ca.* 100–150 Å from the virus bilayer; they form oligomers, the formation of which is essential for intravesicular transport to the membrane surface after biosynthesis, and most importantly, they contain a fusion peptide in a membrane-anchored polypeptide chain [172, 193] (Tables 1 and 2). Typically, these peptides are short segments (up to some 20 amino acids) composed of relatively hydrophobic residues which can be found in N-terminal or internal positions along the transmembrane glycoproteins (*see* references in Table 1) and which are thought to penetrate into the target membrane to cause fusion [43, 79, 137, 139, 140] (*see also below*). Many viral fusion glycoproteins need to be cleaved enzymatically to acquire their fusion capacity; this proteolytic cleavage is a late event in the biosynthetic pathway and occurs near or at the surface of the host cell. Such processing creates a new amino terminus on the transmembrane subunit, resulting in the exposure of the fusion peptide at this N-terminal position [10, 39, 82, 88, 108, 124, 147, 155, 185, 197]. One of the exceptions to this rule is the *env* glycoprotein of Rous sarcoma virus, whose processing does not lead to exposure of an amino-terminal hydrophobic region and whose fusion peptide is internal [91]. Fusion proteins that do not require proteolytic cleavage to express their fusion properties include the G protein of rabies and vesicular stomatitis viruses [64], the E spike of the tick-borne encephalitis virus [152] and the E protein of the Semliki forest virus [101, 161].

Further activation, probably to position the fusion peptide near the target membrane, is accomplished through conformational changes in the fusion protein, induced either by exposure to low pH or as a consequence of virus-cell binding. Fusion of viruses that enter

Table 2. Structural properties of viral and cellular fusion peptides

Virus or cell	Fusion peptide	Length	pH dependence	Secondary structure[a]	Orientation[b]	References for structure and orientation
Influenza virus*	N-terminal HA2	24	low pH	α helix	oblique	[79, 109, 115, 179]
				β-sheet	/	Gallagher et al., 1992[c]
SFV	Internal E1	23	low pH	probably not α helix	n.d.	[101]
TBE virus	Internal E	?	low pH	β-sheet (?)	/	[75, 152]
Rabies virus	Internal G	?	low pH	α helix (?)	n.d.	[43]
VSV	Internal G	21	low pH	α helix (?)	n.d.	[43, 57]
LCMV	Internal G2	23	low pH	α helix (?)	n.d.	Glushakova et al., 1992[d]
Baculovirus	Internal gp64	6	low pH	n.d.	n.d.	Monsma and Blissard, 1995[e]
HIV*	N-terminal gp41	30	neutral pH	α helix	oblique	[104, 117, 123]
				α helix and β-sheet	/	[135]
SIV	N-terminal gp32	21	neutral pH	α helix	oblique	[86, 118, 119]
RSV	Internal gp37	16	neutral pH	n.d.	n.d.	[91]
BLV	N-terminal gp30	?	neutral pH	α helix (?)	oblique (?)	[185]
Sendai virus	N-terminal F1	32	neutral pH	α helix	oblique	[151]
NDV	N-terminal F1	36	neutral pH	α helix	oblique	[12]
Measles virus*	N-terminal F1	19	neutral pH	α helix	oblique (?)	[12, 13]
				β-sheet	/	[47]
HBV	N-terminal S	23	neutral pH	β-sheet	/	[154-156]
sperm*	Internal fertilin α	22	neutral pH	α helix	n.d.	[7, 122]
				β-sheet	/	[132]
	Internal bindin	18	neutral pH	α helix and β-sheet	n.d.	[183]

Model fusion peptides	Length	pH dependence	Secondary structure[a]	Orientation[b]	References for structure and orientation
GALA	30	low pH	α helix	perpendicular	[71, 176]
SFP	14	low pH	α helix	n.d.	[148]
poly(Glu-Aib-Leu-Aib)	⩾18	low pH	α helix	n.d.	[106]
Ac-$(LARL)_3$-NH-CH_3	12	neutral pH	α helix	n.d.	[110]
amphiphilic model peptide	51	neutral pH	α helix	n.d.	[206]
WAE	11	neutral pH	α helix	almost perpendicular	[143, 144, and unpublished observations]

[a] Determined by circular dichroism and/or Fourier transform infrared spectroscopy, in the presence of lipid vesicles. [b] Refers to the angle formed between the peptide molecule and the bilayer surface, assuming an α-helical conformation of the peptide [13]. [c] Gallagher et al., 1992. *Cell* **70**:531. [d] Glushakova S.E. et al., 1992. *Biochim. Biophys. Acta* **1110**:202. [e] Monsma S.A. and Blissard G.W. 1995. *J. Virol.* **69(4)**:2583-2595. * Virus or cell for which the secondary structure of their fusion peptide is questioned or controversial. (?) indicates that the secondary structure or orientation have been predicted by computerized models or deduced from indirect experiments. n.d., not determined.

the cell via receptor-mediated endocytosis occurs in the endosomal compartment, triggered by a mild acidic pH. Upon lowering pH, the influenza hemagglutinin dramatically changes conformation, resulting in a *ca.* 100 Å-projection of its fusion peptide toward the target membrane, as revealed by Bullough and coworkers [17], based upon the crystal structure of a soluble proteolytic fragment of hemagglutinin. Low pH-induced conformational changes in the E glycoprotein of the TBE virus have been deduced from the crystal structure of a solubilized protein fragment [152] and from mapping experiments with monoclonal antibodies [80]. The positions of epitopes that are substantially affected by low pH-treatment lie near the putative fusion peptide. In response to low pH, the E glycoprotein possibly projects up from the viral membrane, lifting the fusion peptide to a position near the target membrane [152, 175]. Such low pH-induced structural changes have also been demonstrated for the SFV E glycoprotein: by swiveling about one another, E1 dissociates from E2 and extends ≈20 Å, probably to position the fusion peptide close to the host cell membrane [61]. Concerning rabies and vesicular stomatitis viruses, knowledge on structural features of the G glycoprotein in its low pH conformation still remains speculative. However, a behavior similar to that of other fusion proteins is plausible [63, 65].

For viruses that do not require an acidic environment to fuse with host cells (and thus fuse directly with the

plasma membrane), activation of their fusion glycoproteins could be triggered by interactions between the fusion proteins themselves or with another protein, and their host cell receptor(s) [69, 82]. For example, the gp160 precursor fusion protein of HIV is cleaved into gp120 (surface subunit) and gp41 (transmembrane subunit containing the N-terminal fusion peptide). Gp120 binds CD4 and a chemokine coreceptor called CCR-5/CXCR-4 on the target membrane [56]. This triggers dissociation of gp120 from gp41 and induces conformational changes in gp41 [170], leading to exposure and penetration of the fusion peptide into the target membrane [6, 20, 188]. A similar activation process is also most plausible for the gp70/p15E complex of Moloney murine leukemia virus [54] and for the Gp2 glycoprotein of Ebola virus [187].

The F protein of paramyxoviruses is absolutely required for fusion, since it contains the fusion peptide [137]. However, increasing evidence strongly suggests that the HN molecule, through its binding to specific receptors on the target membrane, is involved in fusion-promoting activity [11, 85, 181]. Furthermore, it was found recently that paramyxovirus-induced cell fusion required the formation of a complex in which HN and F are physically associated [180, 203]. It can thus be hypothesized that HN binding triggers conformational changes in F, leading to the proper exposure of the fusion peptide near the target membrane. Most recently, it was demonstrated that the binding of a soluble retroviral fusion protein itself to its specific receptor in solution led to the transformation of its ectodomain into an hydrophobic entity able to bind target membranes [32, 83].

The oligomeric organization of the spike proteins of the influenza virus (HA), of VSV (G), SFV (E), of TBE virus (E) and of the paramyxovirus Sendai (F) is known. For HA [197], G [107], and SFV E [102], a trimeric structure has been demonstrated, either as a homotrimer for HA and G, or a heterotrimer E1/E2/E3 for SFV E. Trimerization occurs rapidly (1–3 min) after synthesis in the endoplasmic reticulum and is essential for proper transport to the Golgi complex [67, 107]. Noncovalent interactions between monomers stabilize the spike structure, particularly at the stem and transmembrane regions of the spike [107]. For the E glycoprotein of SFV, intersubunit stabilization is located in the transmembrane regions of E1/E2. TBE virus E spike protein forms a dimer [152]. The fusion protein F of Sendai virus is arranged as a tetramer, consisting of two identical dimers [164]. The oligomeric structure of the HIV gp120/gp41 complex is still controversial, most likely a trimer [188] or tetramer [45]. Note that these proteins also arrange in higher-order oligomers in a 'super-organization,' i.e., these oligomeric structures further oligomerize at the onset of the fusion process, in trimers or tetramers for HA [34], in trimers for the E1/E2/E3 proteic complex of SFV [101] and most probably for the TBE virus E glycoprotein [175] (*see below*).

Cell-Cell Fusion

Recently, a candidate fusion protein involved in mammalian sperm-egg fusion has been identified [7]. It belongs to the ADAM family (proteins containing A Disintegrin And Metalloprotease) and is called fertilin [167]. It shares several biochemical characteristics with viral fusion glycoproteins: (i) it is synthesized as a precursor which is proteolytically processed into α and β subunits [200], which (ii) have the bulk of their mass external to the plasma membrane and (iii) form higher-order oligomers; (iv) the β subunit contains a disintegrin domain capable of binding to an egg integrin and most importantly, (v) the α subunit contains a relatively hydrophobic internal sequence that fulfills the criteria of a candidate fusion peptide [7, 136, 199]. By analogy with several viruses, it can be hypothesized that an interaction between the β subunit and an egg integrin could trigger conformational changes in the α subunit, leading to the upward 'lifting' of the fusion peptide toward the target membrane.

Another ADAM protein, meltrin α, is thought to be involved in the formation of myotubes, resulting from fusion between myoblasts, and the presence of a candidate fusion peptide in its sequence has been predicted. This peptide seems to share some sequence homology with the fusion peptide of Sendai virus [200, 201].

Intracellular Fusion Events

This issue will be only briefly discussed here, since it is beyond the scope of this review. Although the question of 'who does what and how' in intracellular fusion remains largely unclear, it is now generally appreciated that a 'fusion machinery' involving several protein partners, operates in numerous intracellular trafficking events [5, 40, 95, 157, 158, 168, 169]. This fusion machinery is composed of three elements: the ATPase NSF (for NEM-Sensitive Fusion protein), SNAPs (for Soluble NSF Attachment Proteins) and SNAREs (for SNAP Receptors). Both NSF and SNAPs are cytosolic proteins, while SNAREs are integral membrane proteins, present on target membrane (t-SNARE) and transport vesicles (v-SNARE). There is no obvious reminiscence between this fusion complex and that of the viral or other cellular fusion proteins. However, the experimental evidence suggests that a specific and stable association between a vesicle and its target membrane is established via an interaction between v- and t-SNARE. Subsequently, α-SNAP and NSF are recruited to the SNARE complex. A prelude to fusion appears to be the NSF-induced hydrolysis of ATP, which dissociates the SNARE complex [157]. More recent evidence suggests [77, 95] that for-

mation, rather than disassembly of the ternary SNARE complexes drives membrane fusion. The formation of these ternary complexes is accompanied by a release of energy which could possibly help overcoming the energy barrier between membranes (*see* Introduction, and [209]). Most recently, Rothman and coworkers [186] presented some evidence, suggesting that v- and t-SNAREs are the minimal machinery required for membrane fusion. It would thus appear that similar features and/or mechanisms may be involved in intracellular and viral fusion events; this is particularly relevant regarding the general topology of fusion proteins or complexes, with their membrane anchors inserted in opposed membranes, thereby pulled together [186].

Penetrating Insights into the Molecular Mechanisms of Protein-Induced Fusion

Probing the Fusion Sequence of Viral Fusion Proteins

Key parameters of the fusogenic region such as primary sequence, hydrophobicity, conformational changes and orientation into target membranes can be studied by a battery of genetic, immunological and biochemical techniques.

Primary Sequence and Hydrophobicity

Oligonucleotide-directed mutagenesis has been widely used to introduce single or multiple base changes into the DNA sequence that codes for the fusion peptide of several viral glycoproteins. For the influenza HA, it has been shown that single amino-acid replacements completely abolish fusion activity [67, 174], or induce modifications in the threshold pH for fusion [162, 173], or impair late fusion events such as pore formation [162]. Viral fusion activity is eliminated when the N-terminal Gly is replaced by Glu in HA2 [67], while fusion activity is maintained with a Gly $\rightarrow$ Ala substitution [174]. This identifies the N-terminal glycine as a key amino-acid in HA fusion function. Replacement of glycine residues by alanine (a more hydrophobic residue) greatly enhanced the fusion activity of the simian virus 5 F protein [87], stressing the importance of hydrophobicity in the fusion function (*see also below* and [194]). For F glycoprotein of paramyxoviruses, replacement of a non polar residue by a charged amino-acid in the fusion peptide sequence totally blocks the fusion activity of the protein, as reported for NDV, as a result of a decreased hydrophobicity of this region, possibly leading to an impaired insertion of the fusion peptide into the target bilayer [165]. The use of this technique also confirmed that the N-termini of SIV gp32 [10] and of MLV p15E [96] were the fusion peptides, and that hydrophobicity of this region plays a pivotal role to allow its insertion into the target membrane [10, 37, 60, 159, 194].

Protein Oligomerization

Mutagenesis in the fusion peptide of HIV gp41 led to the identification of two glycine residues critical for syncytium formation and virus infectivity [37]. These residues are thought to play a direct role in the fusion activity of the peptide itself, probably through the formation of a 'glycine strip' involved in the oligomerization of several fusion peptides that may participate in the formation of late fusion structures (fusion pores) [37, 76, 194]. However, alterations in fusion peptide oligomerization, syncytium formation, and virus infectivity (even in the presence of excess wild-type gp41) were also obtained by a Val to Glu substitution at position 2 of HIV gp41 [59] or by sequential deletion of amino acids at the N-terminus of gp41 [159]. This indicates that several features (presence of certain amino acids at a given position, hydrophobicity, 'glycine strip,' total number of residues in the sequence) are indispensable for the process of oligomerization and thus for fusion.

Conformational Changes

Mutagenesis studies of the G glycoprotein of VSV [112], and of the E glycoprotein of SFV [101, 11] revealed the involvement of spatially separated regions in the fusogenic activity, and amino acids substitutions in the putative internal fusion peptide resulted in altered or abolished low pH-dependent membrane fusion activity, due to altered conformational properties [57, 58, 208]. Monoclonal antibodies that recognize the native structure or the fusion-competent form of a fusion protein can be used to define which epitopes are lost, rearranged or exposed during the fusion process. Such studies revealed that the region around the fusion peptide of influenza hemagglutinin, buried in the stem domain at neutral pH, become exposed after low pH-induced irreversible conformational change [35, 89, 90, 189, 195, 197], which was recently confirmed by a heat-induced denaturation approach [19]. Similarly, low pH-induced changes in conformation and outward projection of the peptide were also demonstrated for other viral proteins [65, 75, 80, 102, 116, 152].

Penetration and Orientation of the Fusion Peptide into Target Membranes

Direct evidence for penetration of the fusion peptide of some viral glycoproteins has emerged from the use of photoactivable lipid probes incorporated in the target

Table 3. Sequences of some synthetic peptides used as models for Influenza hemagglutinin-induced fusion

Sequence and name of synthetic peptide		Fusogenicity[a]	α-helix[b]	References
GLFGAIAGFIEGGWTGMIDG*	A/PR/8/34 strain	+++ (pH 5)	+ (CD) +++ (IR)	[177] [115]
GLF**E**AIA**E**FIEGGW**E**G**L**I**E**G	peptide III ; E5	++ (pH 5)	+++	[92, 133, 177]
GLF**E**AIA**E**FI**P**GGW**E**G**L**I**E**G	E5P	−	± (α > random)	[133]
GL**EE**FAI**EA**FIEGGW**E**G**L**I**E**G	peptide VII	−	− (β-sheet)	[177]
GLFGAIAGFIENGWEGMIDG*	X31strain, 20-residue	+++ (pH 5)	+++ (CD)	[18, 48, 49, 150, 190]
GLFGAIAGFIENGWEGMIDGWYG*	X31strain, 23-residue	++++ (pH 5) ++++ (pH 5)	++++ (CD) ± (α > β)	[190] [73]
GLF**E**AIAGFIENGWEGMIDG	G4E ; E4	++++ (pH 5)	++++ (CD)	[49, 150, 190]
ELFGAIAGFIENGWEGMIDG	G1E ; E1	±	± (α > β)	[18, 150, 190]
ELFGAIAGFIENGWEGMIDGWYG	G1E	−	− (β > α)	[73]
LFGAIAGFIENGWEGMIDGWYG	ΔG1	−	− (β >> α)	idem G1E
GLFGAIAGFIENGWEG**C**	HA2.17	−	n.d.	[44]
ELFGAI**C**	HA2.7mu1	−	n.d.	[44]
ALFGAIAGFIENGWEG	$G1_2A$	±	n.d.	[174]
GFFGAIAGFLEGGWEGMIAG*	B/Lee/40strain; H-20	++ (pH 5 and 7)	+++	[29, 109]
GFFGAIAGFLEGGWEG	H-16	±	+	[109]

[a] pH values where maximal fusion activity is obtained. [b] Secondary structure determined in the presence of liposomes at the pH value under a. * Synthetic peptides whose sequence corresponds to that of the wild-type fusion peptide in the viral glycoprotein. CD, circular dichroism ; IR, infrared spectroscopy ; n.d., not determined.

membrane (usually liposomes) [16]. Harter and coworkers applied this subtle technique to bromelain-treated influenza HA (BHA), i.e., to the water-soluble ectodomain of HA. They demonstrated that the low-pH induced interaction of BHA with liposomes was mediated by the BHA2 subunit [78]. Further evidence was obtained recently, indicating that the N-terminus of HA2 was the only membrane-inserted region after low pH treatment of intact virus [42, 139]. Hydrophobic photolabeling was also successfully employed to identify a putative fusion region in the G glycoprotein of rabies and vesicular stomatitis viruses. Labeling of G in the presence of liposomes was maximal under low pH conditions, and the insertion of the ectodomain was found reversible [43, 140]. For paramyxoviruses, the only demonstration of the penetration of F glycoprotein into the target membrane was obtained for Sendai virus incubated with liposomes [137]. During early stages of fusion, hydrophobic labeling is almost entirely confined to the F glycoprotein, and more specifically concentrated in the F1 subunit which contains the fusion peptide. Finally, site-directed mutations in SIV gp32 [86] and in BLV gp30 [185] were found to induce alterations in the angle of insertion of the fusion peptide in the target bilayer; in wild-type glycoproteins, the fusion peptide adopted an oblique orientation, whereas the fusogenic domain of mutant glycoproteins laid roughly parallel to or inserted perpendicularly into the target membrane, leading to a reduced fusogenic activity. In summary, several pieces of evidence demonstrate that the known or putative fusogenic peptide from viral fusion proteins plays a crucial role in the fusion process, through its sequence, position in the viral protein under fusogenic conditions and structural features. However, a detailed molecular insight into the involvement of these fusogenic segments and into the early events that trigger viral or cellular fusion may be difficult to obtain using the complex system of an intact virus or a whole cell. Therefore, synthetic peptides corresponding to the putative fusion sequences of viral or cellular fusion proteins, or nonviral related *de novo* synthesized peptides may prove to be highly useful in determining the requirements for particular amino acid sequences, for structural characteristics, and to determine the way fusion peptides may interact with, penetrate into and destabilize a lipid bilayer (*see* Table 2). The use of synthetic peptides can also provide information on the minimal molecular requirements for protein-mediated membrane fusion, and, in conjunction with the use of artificial membranes, offers the possibility to study the influence of lipid species on fusion by modulating the composition of the peptide-interacting membranes.

Synthetic Peptides: Applications and Limitations as Models for Viral and Cellular Fusion

Specific Amino Acids and Hydrophobicity

The peptides corresponding to the putative fusion sequences of viral or cellular fusion glycoproteins are rich in glycine and alanine residues, and their sequence is

Table 4. Sequences of synthetic peptides used as models for viral and cellular protein-induced fusion

Sequence and name of synthetic peptide		Fusogenicity	α-helix	References
GGYCLTRWMLIEAELKCFGNTAV*	Lassa arenavirus FP	+++ (pH 4.5)	probable	Glushakova et al., 1992 (see legend to Table 1)
AVGIGALFLGFLGAAGSTMGAASMTLTVQAR*	HIV-1gp41,LAV_{mal}strain,31-residue	nd	nd	[62, 193]
AVGIGALFLGFLGAAGSTMGAAS*	LAV_{ma1} strain, 23-residue ;	+	+++	[149]
	HIV_{Ala}	+	− (β sheet)	[146]
A**E**GIGALFLGFLGAAGSTMGAAS	HIV_{E2}	−	+++	[146]
AVGIGALFLGFLGAAG	P16 ; HIV16aa ; SPwt	+++	+++	[117, 123, 166]
ALFLGFLGAAG	P11	+	?	[166]
FLGAAG	P6	−	?	[166]
AVGIGALFLGFLG	SPwt.13	+	?	[123]
A IGALFLGFLGAAG	SP-2	++	+++	[123]
A GALFLGFLGAAG	SP-3	±	+++	[123]
A ALFLGFLGAAG	SP-4	−	+++	[123]
AVGIGALFLGFLGAAGSTMGARS*	LAV_{1a} strain ; HIV_{Arg}	++	+ (α > β)	[149]
		++	− (β sheet)	[135]
AVGIGALFLGFLGAAGSTMGARSMTLTVQARQL	WT	+++	+ (α > β)	[104]
A**E**GIGALFLGFLGAAGSTMGARSMTLTVQARQL	V2E	−	++	[104]
GVFVLGFLGFLA*	SIVgp32, SIV_{mac} BK28 strain, 12-residue ; SIVWT12aa	+++	++	[30, 47, 118, 119]
GVFVLGFLGFLATAGS*	idem, 16-residue ; SIVWT16aa	++	+	[118]
GVFVLGFLGFLATAGSAMGAASLT*	idem, 24-residue ; SIVWT24aa	±	++	[118]
GVFVLG**LF**GFLA	SIV7L8F	++	− (β > α)	[119]
GVF**GVAL**L**FLGF**	SIVmutV	−	− (β > α)	[30, 119]
FFGAVIGTIALGVATSAQITAGIALAEAREAKR*	Sendai virus F1 ; WT	+	++++	[151]
FFGAVIGTIAL**A**VATSAQITAGIALAEAREAKR	G12A	+++	++++	[151]
FAGVVLAGAALGVA**A**AAQI	Measles virus F1 fusion peptide	+++	− (β sheet)	[48, 205]
MENITSGFLGPLLVLQAGFFLLTR*	HBV S fusion peptide	+++ (pH 5)	− (β sheet)	[154-156]
HPIQIAAFLARIPPISSIGTCILK*	Sperm fertilin α peptide	+++	++	[122] and Martin I., personal communication
		+++	− (β >> α)	[132]
LGLLLRHLRHHSNLLANI*	Sea urchin sperm bindin, 18-residue B18	+++ (pH 7.4)	++	[183]

Legend appears as in Table 3.

conserved within but not between virus families (Tables 3 and 4, and refs therein). Substitution or deletion of these residues in synthetic peptides led to a similar conclusion obtained by genetic engineering of viruses, that glycine residues play a key role in maintaining proper levels of fusion: replacement of the *N-terminal* glycine or deletion of this residue [18, 44, 73, 150, 174, 190] resulted in a total loss of fusogenic activity toward artificial membranes (Table 3) or reduced hemolytic properties [174]. By contrast, replacement of *internal* glycine(s) affected the peptide fusion activity to a lesser extent [49, 150] or even resulted in a substantial increase in fusogenicity in a peptide derived from the Sendai virus F1 fusion peptide [151]. The functional consequences of these changes are discussed in terms of secondary structure (*see below*, and Tables 3 and 4) and of hydrophobicity (*see above* for viral proteins). It thus evolves as a general rule that substitution of an apolar or polar amino acid for a residue of similar nature hardly affects the fusion properties of the peptides (Tables 3 and 4, and refs therein). By contrast, substitution for a residue of opposite nature leads to a partial or complete loss of activity. This points to the importance of specific amino acids in the peptide sequence for optimal fusion function [37].

Length of Peptides

Typically, fusion peptides in viral or cellular fusion proteins are composed of 20–25 amino acids on average (Tables 2, 3 and 4), a length that would allow membrane spanning. Actually, it appears that shortening of pep-

tides leads to a diminished interaction with lipid membranes and consequently, to diminished or abolished fusion activity (Tables 3 and 4, and references therein). Effective membrane binding of model peptides, adopting an amphipathic α-helical structure, was observed for a minimal length of 8 residues [125]. By striking contrast, the fusion activity of peptides derived from SIV gp 32 was found to be inversely related to the length of the peptide (Table 4, peptides SIVWT12aa, SIVWT16aa and SIVWT24aa; [118]), in spite of conserved structural features. This surprising result is discussed in terms of orientation of the peptide into the lipid bilayer, and leads us to focus in the following on the requirement for secondary structure.

Secondary Structure and Orientation of Fusion Peptides into Membranes

Secondary Structure

Evidence for penetration of the HA2-hydrophobic fusion peptide into the target membrane was first produced from hydrophobic photolabeling experiments on a solubilized fragment of HA by Harter and coworkers [78, 79]. As the average spacing between consecutive labeled amino acid side chains was 3–4 residues, it was suggested that this hydrophobic segment adopted an α-helical structure, necessary for insertion into the bilayer [79]. A similar behavior had previously been reported by Lear and DeGrado [109] with a 20-residue synthetic peptide derived from the fusogenic region of HA2. Interestingly, a 16-residue peptide with no fusogenic activity was found to adopt mainly an extended β-structure. Taken together, these results suggested that (i) hydrophobic interactions could play a dominant role in secondary structure formation, and that (ii) helix formation was required for fusion activity of influenza HA. Since then, increasing evidence has been obtained that indicates a correlation (albeit not strict) between helicity of synthetic fusion peptides derived from a variety of viral or cellular glycoproteins and their ability to interact with and destabilize a membrane (Tables 3 and 4; references therein and I. Martin, *personal communication* on fertilin α). Further support for the view that the α-helical structure could be important for fusion was gained from studies with selectively modified sequences of peptides derived from viral glycoproteins, or with *de novo* synthesized peptides (Table 2 and references therein). Substitution of a proline (a strong α helix breaker; [28]) for a Glu residue in the middle of the sequence of an HA2-derived peptide resulted in a marked decrease in the α helical content, concomitant with an almost complete loss of fusogenicity [133]. Introduction of proline residues in the 12-residue sequence of model amphipathic fusogenic peptides, causing the disruption of the α helix structure, also abolished their fusion activity toward liposomes [110]. Interestingly, the α helical content was gradually reduced by increasing the number of Pro residues in the peptides from one to three. It must be noted however that this α helix-breaking propensity of Pro residues is not a general rule, since the putative fusion peptide of sperm fertilin α displays a high α helical content in the presence of liposomes, in spite of the presence of three prolines (*personal communication;* I. Martin, Table 4). Moreover, the presence of the Pro-Pro sequence is required for efficient fusion activity [136]. Interestingly, a peptide fragment derived from the binding domain of the sea urchin acrosomal protein binding, also adopts an α-helical structure, when triggering fusion of SM/cholesterol liposomes. Formation of an α-helical structure requires the presence of Zn^{2+} ions which interact with His residues in the peptide according to a Zn-finger principle, thus giving rise to the formation of oligomeric complexes [183]. Finally, high α-helical contents were also observed for a model fusion peptide, WAE, and an analogue with a Pro to Leu substitution [144]. Hence, the impaired fusion activity of the WAE Pro ''mutant'' was attributed to increased hydrophilicity of this peptide, due to the Pro residue.

Similarly, perturbation of the segregated distribution between hydrophilic and hydrophobic residues (amphipathic character) of an α helical peptide derived from HA2 (compare peptides III and VII in Table 3) led to disruption of the α helical structure in favor of a β-structure, and was accompanied by a loss of fusogenicity [177]. However, it has also been shown that elimination of the amphipathic character of the α helix in the model fusion peptide WAE, without affecting the high α helical content as such, resulted in enhanced membrane destabilizing properties, in terms of fusogenicity and ability to cause vesicle leakage [143, 144]. An intermediate behavior was observed for the pH-dependent fusogenic model peptide GALA and its counterpart LAGA: both peptides exhibited a random coil to α helix transition when lowering the pH. But only GALA initiates membrane fusion, due to the amphipathic character of its α helical conformation at low pH, which is abolished in LAGA [141] (*see also* Table 2 and references therein). Hence, it appears that helicity is necessary but seemingly not sufficient for fusion, as will be further discussed in the following.

A complete loss of fusion activity, related to a prevalence of β-over α-structures was obtained for an HA2-derived peptide, caused by a single substitution of the N-terminal amino acid (G1E, Table 3) or by its deletion (ΔG1, Table 3) [18, 73, 150, 190]. However, the existence of a direct relation between helicity and fusogenicity was questioned by Gray and coworkers [73], who suggested that fusogenicity correlates weakly with helic-

ity, but strongly with the α/β secondary structure ratio. Indeed, neither was a direct relation between fusion and α-helix structure found for model 11-mer fusion peptides, and apart from a potential relevance of the α/β ratio, a prominent role for peptide orientation toward the target membrane was also suggested [144]. However, differences in the estimates of secondary structures may evolve from differences in the experimental conditions (CD or IR spectroscopy, size and composition of the vesicles used, lipid-to-peptide ratio), and may lead to erroneous interpretations (*see below*).

More recently, several lines of evidence have also suggested that some viral or cellular fusion peptides could adopt β-structures under fusogenic conditions. Studies were conducted with a 23-residue synthetic peptide derived from HIV-1 gp41 (HIV_{Arg}, LAV_{1a} virus strain; Table 4) [135]. Under nonfusogenic conditions, HIV_{Arg} adopted an α helical conformation, and through clustering of several helices, induced leakage of liposomal contents due to pore formation. By contrast, under fusogenic conditions, the peptide adopted an antiparallel β-structure. Similarly, using a 23-residue peptide derived from LAV_{ma1}-strain HIV-1 gp41 (HIV_{Ala}; Table 4), Pereira et al. [146] showed a prevalence of β-structures over α-helical conformation in the same model system; moreover, a peptide displaying a V → E substitution at position 2 was found to be unable to fuse membranes (*see also* [59]), while adopting exclusively an α helical conformation. These demonstrations that β-structures are likely involved in peptide-induced membrane fusion call however for some remarks concerning the experimental conditions: in both studies, peptide was added to lipid vesicles in a low lipid-to-peptide ratio (L/P = 65), which might account for the observed low amount of α-structures. Indeed, numerous studies using different lipid-to-peptide ratios indicated that the α helical content increases when increasing this ratio [98, 105, 106, 115, 118, 119, 123, 149, 150, 206, and our unpublished observations]. Moreover, in studies where an excess of lipid vesicles was used, such that essentially all the peptide would bind, it was demonstrated that a maximum in the lipid-bound peptide fraction was not obtained for L/P ratios below *ca.* 100 [104, 109, 149, 151]. From this, it is evident that only at high L/P values, the peptide's ability to insert into and destabilize the lipid bilayer as an α helix becomes optimal, whereas at lower L/P ratios, the peptide is essentially bound to the surface, largely as aggregated antiparallel β-sheets [109, 118, 149]. This has also been demonstrated for signal peptides which display α helical structures when inserted into membranes and β conformations when surface-bound [14]. It must also be noted that spectroscopic measurements as carried out by Nieva et al. [135] and Pereira et al. [146] were recorded under equilibrium conditions, i.e., after a 30-min incubation of peptides with vesicles when fusion has been completed. The possibility that the α helix conformation is involved at an early stage of fusion can therefore not be excluded [135].

Synthetic fusion peptides derived from sperm fertilin α [132], measles virus F1 [48] and from the S protein of hepatitis B virus [154, 156] were also found to adopt preferentially a β-sheet conformation in a lipid environment. However, a note of caution concerning the experimental conditions as indicated above also holds in these cases. Structure determinations were performed by circular dichroism on small lipid vesicles and by infrared spectroscopy on large vesicles without separation of the unbound peptide from the inserted one. This also calls for some remarks: the use of CD is often precluded by the scattering effect of liposomes in suspension at wavelengths that would allow reliable estimations of α- or β-structures; CD experiments are thus performed at low L/P ratios, raising the above limitations. Also in this case, a significant proportion of the peptide will not be inserted into the bilayers, possibly leading to an underestimation of the α helical content and overestimation of the β structures. A second point concerns the use of SUV. As convincingly shown by Rafalski et al. [150], SUV are less able to discriminate between fusion active or defective peptides than LUV, and have a higher potential to interact with hydrophobic peptides due to their smaller radius of curvature [198]. This must also be considered in the light of the low L/P ratio (= 60), as used by Muga et al. [132]. This limitation was bypassed by using LUV for IR measurements; but (as for SUV) without distinguishing between unbound and inserted forms of the peptide, the observed prevalence of β-structures, possibly due to the aggregated surface-bound population, is readily explained. Indeed, Lüneberg et al. [115] demonstrated for an HA2-derived peptide that elimination of the unbound peptide by gel filtration led to an almost complete disappearance of β-sheet structures in favor of α helix (*see also* [118, 119] for SIV gp32-derived peptides; E.I. Pécheur and I. Martin, *unpublished observations* on WAE model peptide and [143]). It appears clearly from the foregoing that peptide penetration into a lipid bilayer to induce membrane fusion relies on the peptide's secondary structure. However, it is also apparent that this structural feature is not sufficient to bring about fusogenicity or complete membrane merging. The need for a certain depth of penetration and orientation of the peptide into the target membrane will now be discussed.

Peptide Penetration into Membranes

Penetration of fusion peptides into membranes can be determined by monitoring changes in intrinsic fluorescence of aromatic amino-acids contained in the peptide sequence (Trp, Tyr or Phe). By doing so, it appears that

for most of the peptides tested, the greater the changes in fluorescence, the higher the peptide's fusogenicity. It was noted for pH-dependent fusion peptides that they were located in a more hydrophobic environment into phospholipid membranes at pH 5 than at pH 7.4 [109, 133, 141, 150, 176]. Specific studies were conducted with lipids brominated at various positions of their acyl chains, to determine the depth of penetration of these peptides into bilayers through quenching of intrinsic fluorescence. Interestingly, these studies showed that rather than penetrating more deeply into the membrane, the peptide undergoing low-pH activation exhibits a change in its conformation or orientation [29]. Using the same experimental procedures, a role for peptide conformation into membranes was also strongly suggested for the GALA model peptide, although its depth of penetration was different at pH 7.4 and pH 5. Thus, the mode of insertion and the orientation of fusion peptides into bilayers appear logically as key features for fusion induction, in addition to the mere secondary structure.

Peptide Orientation into Membranes

Advances in this field have come from the pioneering studies of Brasseur and his colleagues. Assuming an α helical conformation, amino acid sequences of viral fusion peptides were submitted to computer analysis and molecular modeling on the basis of their hydrophobicity profiles [12, 13]. Due to the calculated asymmetry of the hydrophobic envelope along the α helix axis, the orientation for these peptides into bilayers was predicted as oblique with respect to the lipid acyl chains. Experimental evidence was then obtained, strongly supporting the validity of this theoretical analysis procedure. Conformational studies using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) were conducted on synthetic peptides derived from HA2, HIV gp41, SIV gp 32, NDV F1, BLV gp30, and revealed that this unusual oblique orientation was adopted by these peptides in a monolayer system [163] and under fusogenic conditions (Table 2, and references therein). A similar conclusion was drawn for the putative fusion peptide of sperm fertilin (I. Martin, *personal communication*). Moreover, a correlation between oblique orientation and fusion activity was established using peptides with modified sequences as compared to the wild-type peptide (Table 4 and references therein). It must be noted that when peptides display an extended β-structure, no orientation can be experimentally determined. Indirect evidence for an oblique orientation of HIV gp41-derived peptides was obtained by Kliger et al. [104], since these peptides were efficiently cleaved by enzymatic treatment, although their N-termini were inserted into the hydrophobic core of the membrane. Note that the results obtained for the orientation of synthetic peptides are entirely consistent with site-directed mutagenesis studies on viral fusion glycoproteins of SIV [86] and BLV [185]. Conversely, lack of fusion activity was related to either parallel [123] or perpendicular orientation with respect to the membrane surface [30, 71, 119]. However, experimental conditions (planar bilayers or vesicles, mode of preparation, state of hydration) could strongly influence the orientation of the peptide, as demonstrated above for secondary structure determinations [92, 93]. Note also that apart from one mutagenesis study conducted on the whole SIV fusion protein [86], other studies use synthetic peptides as *free monomers in solution*, which contrasts their normal membrane-anchored environment. Recently, we observed an almost perpendicular orientation (with respect to membrane surface) into target bilayers of the membrane-anchored model fusion peptide WAE (E.I. Pécheur, I. Martin and D. Hoekstra, *unpublished observations*), which closely resembles a viral fusion peptide in its membrane-associated environment [143, 144]. A difference in the fusion peptide orientation into target membranes was also noted between a solubilized fragment of HA and HA on intact virus (compare [79] with [171]). A tilted conformation of the fusion peptide as a consequence of the tilted conformation adopted by whole HA trimers has been observed [179], and the HA fusion peptide was found to deeply modulate the global orientation of the HA2 subunit [72]. This emphasizes the importance of membrane anchorage of fusion glycoproteins and/or peptides in the overall fusion process, probably to convey an adequate orientation.

Role of Membrane Anchorage in the Fusion Process

Studies using synthetic peptides as described in the foregoing were conducted with peptides in solution, i.e., a relatively poor mimic of the membrane-attached fusion peptides in vivo. Moreover, a function in membrane aggregation, a necessary condition for fusion, is thought not to be contained in the fusion peptide. Although useful information and results consistent with the «in vivo» behavior have been obtained with 'free' peptides on requirement for certain amino acids, secondary structure and orientation (*see above*), no conclusion can be drawn on the involvement of anchorage in the fusion process. It is easily conceivable that membrane anchorage influences the secondary structure and orientation of peptides or proteins through mobility restrictions and geometrical constraints, and thus influences fusion induction [84, 176]. A model system, in which a 15-residue peptide derived from GALA and covalently anchored to a liposomal surface triggered fusion with target LUV at acidic pH (Table 2, SFP; [148]) more closely simulates the behavior of a membrane-bound protein. The amount of

α helical structure increased with decreasing pH, and could be directly related to the fusion activity. However, the fusion process was found leaky. More recently, an 11-mer amphipathic synthetic peptide called WAE was observed to induce a non-leaky fusion process in an LUV model system at neutral pH, provided that the peptide was membrane-anchored [143]. Thus its fusogenic properties strongly depend on mobility constraints, since the free form of the peptide was unable to induce fusion. Interestingly, the membrane-anchored form displayed a high α helical content, whereas peptide in solution adopted essentially β-pleated structures. This strongly suggests that membrane anchorage could govern (at least partly) the ''controlled'' conformational changes from β- to α-structures that lead to fusion [144]. To our knowledge, this is the first demonstration of a direct correlation between membrane anchorage and secondary structure formation for peptide-induced fusion. Note that this agrees well with the view that fusion peptides would penetrate membranes as sided insertional helices [79].

In fact, most of the studies demonstrating the close relationship between membrane anchorage and fusogenicity were conducted on viral glycoproteins. The evidence was provided by studies [68, 191] in which cells were infected with a recombinant virus producing a HA lacking its hydrophobic C-terminal anchor. At these conditions, cell-cell fusion activity did not occur after trypsin treatment and exposition to low pH. Similar results were obtained after exposing the cells to the bromelain-cleaved soluble HA fragment BHA, or to HA rosettes in which the HA molecules remained associated to one another through their hydrophobic tails. This clearly indicates that the fusion activity of HA depends on its attachment to a (lipid) matrix, i.e., either the viral envelope or the plasma membrane. The question thus arises whether the viral protein requires to be anchored to or embedded into the membrane to exert its fusogenic properties. Of interest in this respect are experiments in which the transmembrane and cytoplasmic domains of HA [100] and of SV5 F protein [4] were replaced by a glycosyl-phosphatidylinositol (GPI) lipid tail. These GPI-anchored proteins induced an incomplete fusion process or hemifusion, i.e., lipid mixing of the outer leaflets of the membranes without coalescence of internal contents. This demonstrates that lipid anchorage is necessary to trigger fusion (in conjunction with the fusion peptide), but that full fusion requires the presence of the transmembrane domain of HA. This study was completed by Melikyan et al. [128], showing that GPI-anchored HA-induced fusion did not lead to pore formation. It was thus suggested that the transmembrane domain was involved in late fusion events through an effect on the inner leaflet of fusing membranes [126]. However, clustering of the viral glycoproteins at the fusion site and the occurrence of late conformational changes leading to proper exposure of the fusion peptide(s) were not evaluated. Hence, with these considerations, the fact that the fusion peptide is probably the only part of the protein which inserts into the target membrane to induce fusion, and that a lipid-anchored peptide can trigger complete membrane merging [143], such model systems consisting of a membrane-anchored fusion peptide would prove useful in dissecting even more subtly the events, actors and parameters of membrane fusion.

It has been claimed that membrane destabilization leading to fusion requires the concerted action of several fusion monomers at the site of fusion. This supposes a lateral mobility of these proteins, conceivably regulated by their membrane anchorage [46]. Hence, the intrinsic density of fusion glycoproteins at the viral or cellular surface plays a pivotal role in fusion promotion [34, 127], leading to the oligomerization into a fusion protein complex acting in cooperative manner in the ultimate membrane merging steps.

Importance of Protein Oligomerization at the Fusion Site

It has been shown that influenza HA-induced fusion requires the cooperative addition of (at least) three HA trimers, possibly reflecting that an oligomeric fusion peptide complex arises at the contact site of membrane fusion [34]. Indeed, pore formation and dilation have been suggested to involve the concerted action of six HA trimers [8]. Similar involvement of protein oligomers in the fusion process was shown for other proteins [15, 59, 102, 164, 175]. However, whether overall protein oligomerization also reflects oligomerization at the level of the fusion peptides at the site of fusion, remains highly speculative [76, 196]. This is due to the fact that the assessment of peptide clustering in the protein complex core is beyond the limit of resolution that can be obtained with the techniques currently used to determine protein structural features. It appears therefore that studies conducted on synthetic peptides (i.e., out of their 'normal' proteic environment) could shed light on this key element in the mechanism of membrane fusion. Arguments in favor of peptide oligomerization at the fusion site could be inferred from the following observations: (i) both rates and extents of fusion events such as lipid and/or internal contents mixing increase when the peptide-to-lipid ratio increases; (ii) most of the peptides studied have a tendency to self-associate in the absence of lipids [104, 109, 142, 176, 190] (note however that this aggregation propensity could also be due to structural features, β-structures displaying a particularly high tendency for auto-association); (iii) aggregation in the membrane-bound state was demonstrated for several peptides [104, 151]; (iv) some fusogenic peptides were

studied for their ability to form pores in the target membrane, which usually involves several peptide molecules [55, 142, 166]. It must also be noted that peptides devoid of fusion activity show impaired aggregation properties, both in solution [104, 109, 141, 190] and in their membrane-bound state [73, 104, 141]. This strongly suggests that peptide oligomerization or clustering is related to optimal fusion. Interestingly, clustering of the model membrane-anchored peptide WAE is only observed under conditions where fusion occurs. However, fusion could occur without detectable peptide oligomerization, as long as proper peptide penetration into the target bilayer can take place. This suggests that peptide oligomerization, although needed for optimal fusion, is not a prerequisite for fusion, and that peptide penetration could be the major trigger in peptide-induced fusion [145].

To summarize the foregoing considerations, the following general conclusions can be reached: (i) for systems (e.g., viruses, sperm) in which the fusion protein has been (tentatively) identified, it appears that membrane anchorage is necessary for fusion glycoproteins to exert their fusogenic properties; secreted or cleaved proteins are inherently nonfusogenic; (ii) a membrane-spanning length of the fusion peptide is not an absolute prerequisite for complete membrane merging [143, 148]; (iii) clustering of glycoproteins at the fusion site is related to optimal fusion. The underlying parameters to these observations are the *proper exposure of the fusion peptide* through late conformational changes, and the *proper peptide/lipid* (*peptide penetration*) *and peptide-peptide interactions.* These latter interactions involve not only the secondary structure and the orientation of the fusion peptide(s), in close relation with membrane anchorage and clustering, but also (and most logically), the ease by which the peptide penetrates into the hydrophobic core of the target bilayer and the ensuing ability of this membrane to undergo a bilayer-to-nonbilayer transition. These issues will be briefly discussed in the following.

Importance of the Molecular Shape of the Peptides

In order to fuse, membranes destined for fusion must bend toward each other to become closely apposed. It is conceivable that bending of the membrane-anchored fusion protein itself, as shown for HA [179] may cause or facilitate the bending of lipids in the target bilayer. Particularly at fusion-active conditions of viruses, they are firmly tethered to the target membrane via the inserted fusion peptides. A similar situation can be considered in the case of SNAREs mediated fusion, where the ternary complex of t- and v-SNARE displays more helical structure and is more folded than any of the individual proteins. A role of these structural features in overcoming the energy barrier normally imposed by membranes that come into close proximity, has been suggested [95]. Such concerted bending action would drive the interacting bilayers into the hemifusion intermediate, involving the formation of a 'stalk' which represents a transient, highly bent lipid intermediate [27]. This evolves from the increase in negative monolayer curvature when the fusion peptide inserts into the bilayer. From this, it becomes evident that the shape of the peptide molecule plays a key role in modifying bilayer curvature [53]. Assuming an amphipathic α helical structure, Brasseur rationalized the lipid association of fusion peptides, based upon their hydrophobicity profiles and the angles subtended by hydrophobic and hydrophilic residues in the helical wheel structure [12]. It appeared that for a number of fusion peptides, the angle between hydrophobic and hydrophilic moments were quite similar (*ca.* 180°), as was also observed for model peptides [103]. Molecular modeling of the peptide shape shows that the cross-sectional shape of peptides that destabilize bilayers by increasing negative curvature strain appears as an inverted wedge, with its apex at the (narrow) polar face and its base at the (broad) non polar face; fusion peptides and lytic peptides such as e.g., melittin, mastoparans, magainins or pardaxin belong to this class of amphipathic helices. It must be noted that lytic peptides, which have a broader nonpolar face than fusion peptides (hydrophobic angle > 200°) display far higher destabilizing properties, since most of them are hemolytic and some are bactericidal in vivo (for a review, *see* [31]). In contrast, the cross-sectional shape of peptides inducing positive curvature strain (and thus stabilizing membranes) appears as a wedge with a large polar apex and a narrow apolar base; amphipathic helices from apolipoproteins fall into this category. This elegant molecular modeling was developed by Epand and his colleagues (*see* [53, 182] for a review), and was called the reciprocal wedge hypothesis. Indeed, by analogy with the molecular shape of phospholipids and their effect on membrane stability [94], the authors hypothesized that amphipathic helices with similar cross-sections could mimic the effects of phospholipids on lipid bilayers. In particular, the membrane-destabilizing properties of fusion peptides, modeled as inverted wedges, can compare with those of inverted cone-shaped phospholipids such as unsaturated phosphatidylethanolamines (PE), cholesterol or monoacylglycerols [22, 25, 182]. Moreover, this wedge-like shape could conceptually be related to a relative ease of the fusion peptide to ''submerge'' into the target bilayer and to induce a bilayer-to-nonbilayer transition. Indeed, we showed for the model peptide WAE that the wider the intrinsic head group spacing at the target membrane level, the more easily the peptide could submerge [145]. The fusogenic activity of this peptide has thus been determined toward target liposomal membranes, composed

of various PC species. Note that the molecular shape of this helical peptide can be modeled as an inverted wedge, with a narrow polar apex formed by three Glu residues and a broad nonpolar base [143]. Indeed, a strong relation between fusion rates and intrinsic phospholipid head group spacing was observed [145]. This is the first demonstration that brings validity to the above hypothesis, and most interestingly, a similar relationship was recently observed for the fusion of Golgi membranes with liposomes of various PC species (*unpublished observations*). It would therefore appear that biological protein-mediated fusion processes could be governed by similar molecular parameters as described above for fusion peptides. Through such experiments, it also emerged that the ease of the *target bilayer* to undergo a bilayer-to-nonbilayer transition could pose as another regulating step in the fusion process. This relative ease could also be related to a molecular shape parameter, at the level of lipids in this case.

Role of Lipids of the Target Membrane in Peptide- or Protein-induced Fusion

The promotion of membrane fusion by unsaturated PE is a well-documented phenomenon, and has been related to the conical shape of the molecule, which leads to the formation of the hexagonal (H_{II}) phase. H_{II} phase formation into a membrane composed of various (phospho)lipids is very unlikely, but intermediates between bilayer (lamellar) and H_{II} organization could provide local and transient departures from the bilayer structure, which suffice to trigger membrane fusion. The pioneering work of Chernomordik and his colleagues has shed light on the nature of these intermediates, called stalks and displaying a net negative curvature [26]. Thus, shifting the spontaneous curvature of monolayers to more negative values by adding lipids that support H_{II} phase formation (due to their molecular shape) should promote stalk formation. Such a promotion has been demonstrated with PE, cholesterol, *cis*-unsaturated fatty acids and monoacylglycerols in a number of membrane fusion models. Concerning viral fusion, Yamada and Ohnishi [202] showed that VSV fusion activity with artificial membranes could be greatly enhanced by adding cholesterol to the target bilayer, and that fusion increased with the number of *cis*-double bonds in the phospholipid acyl chains. *Cis*-unsaturated phospholipids have a higher tail-to-head volume ratio than trans-unsaturated or saturated phospholipids. This is due to the formation of a kink in the acyl chain by the *cis*-double bonds, expanding the center of the bilayer and favoring the formation of highly-bent intermediates [50, 178]. Fusion promotion by cholesterol was also found for the fusion of Sendai virus [21], Sindbis virus [160] or influenza virus with liposomes [138]. In a similar manner, unsaturated PE was found to promote fusion of liposomes with synthetic peptides [120], with enveloped viruses [1, 99, 114, 160, 192], with reticulocyte endocytic vesicles [184], and with sea urchin egg cortical granules [22]. PE also promotes fusion between endoplasmic reticulum-derived vesicles and Golgi membranes [131]. Finally, *cis*-unsaturated fatty acids promoted fusion of endosomes, microsomes, and chromaffin granules [22], while also baculovirus-mediated [24] and influenza-mediated cell-cell fusion [25] were stimulated. Note that, conversely, lysolipids that display a cone shape complementary to that of PE inhibit fusion by increasing positive curvature strain (reviewed in [22, 23]). From the foregoing, it therefore appears that: (i) a common molecular mechanism is involved in viral and cellular fusion processes; (ii) this mechanism involves an increase in negative curvature strain that leads to stalk formation; (iii) any compound of *lipidic or proteic nature*, susceptible to promoting negative curvature strain, will concomitantly promote membrane fusion.

Modifications of Bilayer Curvature: Consequences for Membrane Fusion

The importance of the orientation of the fusion peptide into the target membrane to cause fusion was stressed in a previous paragraph. It appears that this feature could also be related to the ability of the peptide to induce either negative or positive bilayer curvature. Fusion peptides from a number of viruses [36, 48, 49, 205] were found to promote the formation of fusion intermediates of negative or inverted (hexagonal-like) phases and to lower the lamellar-to-hexagonal phase transition temperature of unsaturated PE lipid films (Table 3). By contrast, nonfusogenic modified peptides (E1 and E4, Table 3) had no effect on this transition temperature. However, for these peptides, differences in the orientation between wt and modified sequences have not been experimentally assessed. A direct relation between orientation and bilayer curvature was established for SIV gp32-derived peptides (Table 4). The SIV wt fusion peptide, inserting at an oblique angle, was fusogenic and able to lower the lamellar-to-hexagonal phase transition temperature [47] by inducing a negative curvature strain [30]. Conversely, the nonfusogenic SIVmutV, displaying perpendicular insertion into membranes, did not facilitate hexagonal phase formation due to its positive curvature-inducing effect [30]. Moreover, in many peptide fusion model systems, addition of lysolipids to the target bilayers results in an inhibition of fusion, as pointed out in the previous paragraph [47, 120–122, 143]. In some cases, the inhibition of fusion could be directly correlated to an effect of lysolipids on the intramembrane orientation of the fusion peptide: a 12-residue SIV fusion peptide adopted an orientation parallel to the membrane surface

in the presence of lysophosphatidylcholine [117]. Conversely, the presence of PE in the membrane allows a proper insertion of an HIV gp41-derived peptide, as compared to virtually no insertion (parallel orientation) in its absence [120]. This provides strong support to the view that a correlation may exist between the orientation of the fusion peptide and bilayer curvature properties (intrinsic, through bilayer composition, or induced by peptide insertion). However, in the foregoing examples, peptides which induced or increased positive curvature were *fusion-inactive;* but can peptides inducing positive curvature *inhibit fusion*, like lysolipids do? A hydrophobic tripeptide resembling the N-terminus of Sendai F1 (Phe-Phe-Gly) was found to inhibit viral infectivity and virus-induced cell-cell fusion, by an interaction of the peptide with the cell membrane [153]. In subsequent work, this tripeptide, Z-D-Phe-L-Phe-L-Gly or ZfFG (in which Z is a carbobenzoxy group), was found to stabilize bilayers in their lamellar phase [51] and to inhibit the formation of inverted phases [99] by increasing ordering in the acyl chain region of the bilayer [33, 204]. This was attributed to its positive curvature-inducing effect, and interestingly, its shape and orientation seemed to be an important feature as well, in spite of its small size. Indeed, fusion inhibition induced by this peptide very much depends on the charged C-terminus at the head group level and on the N-terminal carbobenzoxy group [52]. A ZfFG complementary peptide was synthesized, with an N-terminal D-Phe exposed to the head group region and a C-terminal Gly blocked by an O-benzoxy group [52]. This peptide, fFGOBz, was an efficient fusion inducer, contrary to the inhibiting effect of ZfFG. It must be noted that the tripeptide sequence Phe-X-Gly is present in fusion proteins (peptides) of all paramyxoviruses in the N-terminal position, of all retroviruses in internal position, and the inverse structure exists in influenza hemagglutinin as Gly-X-Phe ([62] and *see* Tables 3 and 4). The exact relevance to fusion of this short segment has not been clearly established, in spite of numerous studies using mutant viruses or modified synthetic peptides (*see above*). In light of the considerations on ZfFG and fFGOBz peptides, one may therefore speculate that this tripeptide motif in viral fusion proteins could play a key conformational and orientational role in order to create the optimal conditions needed to perturb and fuse bilayers.

Perspectives

The data discussed in the previous section demonstrate that shape, orientation and effects on bilayer curvature of fusion peptides are intimately related to each other, and reveal an unexpected similarity between lipids and peptides in their mode of action at the molecular level. Studies involving synthetic peptides have revealed the importance of these issues. However, it is apparent that synthetic peptides can only partly mimic the overall mechanism as to how a viral or cellular protein induce membrane fusion. For example, unlike a carefully controlled process like intermembrane attachment as in the case of viruses and cellular membranes, fusogenic peptides do not interact with specific receptor(s) on the target membrane. Moreover, the involvement of other segments of the fusion proteins in the fusion process cannot be assessed by using such peptides, being ''released'' from their normal environment. Besides, most of the peptides used in solution are able to form a *cis*-complex between (lipid) molecules within the same plane of the bilayer, but not a *trans*-complex, needed to bring adjacent bilayers into close apposition. These peptides thus lead to membrane destabilization, accompanied by leakage of internal contents. To date however, fusogenic peptides, and particularly membrane-*anchored* peptides, have provided precious information on structural requirements for fusion (amino acid specificity, secondary structure, orientation, molecular shape) and have given insight into the molecular nature of several steps of the overall fusion process. Concerning their use, the conclusion appears justified that structure and function of fusion proteins are closely related.

With the perspective of gaining further insight into these molecular features, the development of model peptide systems resembling more closely the membrane-anchored environment of a fusion protein (density, anchorage, oligomerization, etc.) seems a most promising challenge for the next few years.

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