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Membrane Destabilization by N-Terminal Peptides of Viral Envelope Proteins

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Summary. The fusion of lipid enveloped viruses with cellular membranes is thought to be mediated by the insertion into the target membrane of the N-terminal polypeptides of viral spike glycoproteins. Since membrane destabilization is a necessary step in membrane fusion, we investigated whether synthetic peptides with amino acid sequences corresponding to the N-termini of influenza virus hemagglutinin (HA2), vesicular stomatitis virus G-protein and Sendai virus F-protein, induce the destabilization and fusion of phospholipid vesicles. Membrane destabilization by the peptides was monitored by the release of aqueous contents of large unilamellar phospholipid vesicles. Aggregation was detected by a resonance energy transfer assay. Membrane fusion was followed by means of assays for the intermixing of phospholipids and of aqueous contents. The 17-amino acid HA2 peptide (HA2.17) destabilized phosphatidylcholine (PC) vesicles even at neutral pH, but the rate and extent of destabilization increased at lower pH. This peptide did not mediate appreciable release of contents from phosphatidylserine (PS) vesicles. HA2.17 induced neither aggregation nor fusion of PC or PS vesicles. In contrast, the 7-amino acid N-terminal peptide of G-protein (G.7) destabilized PS-containing membranes and not pure PC vesicles. Although G.7 caused aggregation of and lipid mixing between PS vesicles, it did not mediate any detectable intermixing of aqueous contents. The presence of cholesterol in PC membranes did not affect the destabilization caused by the N-terminal peptide of Sendai virus F-protein (F1.7), suggesting that cholesterol is not necessary for the effective interaction of this peptide with membranes, contrary to earlier proposals. Our results support the hypothesis that the hydrophobic N-terminal region of certain viral envelope proteins insert into and destabilize target membranes.

Key Words membrane fusion · fusion peptides · lipid enveloped viruses · phospholipid vesicles · influenza hemagglutinin

Introduction

Lipid enveloped viruses infect their host cells by fusing with either the plasma membrane, as in the case of Sendai virus, or the endosome membrane after endocytosis of the virion (Ohnishi, 1988; Okada, 1988; Hoekstra & Kok, 1989; Marsh & Helenius, 1989). The former process takes place at neutral pH, whereas the latter requires the acidification of the endosomal lumen. Membrane fusion is mediated by membrane glycoproteins of the viruses (White, Kielian & Helenius, 1983; Düzgüneş, 1985; Spear, 1987; Ohnishi, 1988; Stegmann, Doms & Helenius, 1989). The homology of the amino acid sequence of the N-termini of these proteins has led to the proposal that this region is intimately involved in the fusion process (Gething, White & Waterfield, 1978). Synthetic peptides corresponding to these sequences can inhibit viral infectivity or fusion (Norrby, 1971; Richardson, Scheid & Choppin, 1980; Kelsey et al., 1990). The 26-amino acid N-terminal peptide from the G-protein of vesicular stomatitis virus (VSV) exhibits pH-dependent hemolytic activity, and the hemolytic domain appears to reside in the 6-amino acid N-terminal region (Schlegel & Wade, 1984*a,b*). The hydrophobic N-terminal peptide of the Sendai virus F-protein is normally exposed on the surface of the protein (Gething et al., 1978; Hsu, Scheid & Choppin, 1981; Asano, Murachi & Asano, 1983), whereas that of the influenza virus hemagglutinin (HA2) is exposed only when the pH is lowered (Skehel et al., 1982; White & Wilson, 1987). A crucial step in membrane fusion is destabilization of the lipid bilayer within the area of adhesion (Düzgüneş, 1985; Chernomordik, Melikyan & Chizmadzhev, 1987; Düzgüneş & Bentz, 1988; Hoekstra & Wilschut, 1989). Thus, destabilization of phospholipid vesicles by N-terminal peptides may provide insights into the fusogenic potential of the envelope protein and the mechanisms of fusion.

We have reported previously that a synthetic peptide (HA2.7) corresponding to the 7 amino acids of the N-terminus of HA2 (X-31 strain) induces conductance fluctuations in planar bilayers and leakage from liposomes at neutral pH (Düzgüneş &

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Table. The amino acid sequence and the average hydrophobicity (Eisenberg et al., 1984) of N-terminal peptides of viral envelope glycoproteins, and the membrane destabilizing effect of the peptides[a]

Peptide	Sequence	Hydrophobicity	Destabilization	
			PC	PS
HA2.7	GLY-LEU-PHE-GLY-ALA-ILE-CYS	0.79	−/+	−
HA2.10	GLY-LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-CYS	0.78	+	−/+
HA2.17	GLY-LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-ILE-GLU-ASN-GLY-TRP-GLU-GLY-CYS	0.51	+ + +	−/+
G.7	LYS-PHE-THR-ILE-VAL-PHE-CYS	0.51	−/+	+ + +
G.11	LYS-PHE-THR-ILE-VAL-PHE-PRO-HIS-ASN-GLY-CYS	0.27	−	−/+
F1.8	PHE-PHE-GLY-ALA-VAL-ILE-GLY-CYS	0.84	+ +	−/+

[a] Destabilization of phosphatidylcholine (PC) or phosphatidylserine (PS) liposomes is indicated by the extent of the release of contents 1 min after the addition of the peptides at pH 7.4. (−): No release (≤1%). (−/+): Less than 4% release. (+): Between 4 and 10% release. (+ +): Between 10 and 20% release. (+ + +): Greater than 20% release.

Gambale, 1988). Mutant peptides in which the glycines at the N-terminus or the 4-position are replaced with glutamic acid are considerably less effective in causing conductance changes or leakage (Düzgüneş & Gambale, 1988), consistent with the impairment of the fusion activity of HA2 molecules with identical mutations (Gething et al., 1986). A similar correlation was observed by Rafalski et al. (1991) who used 20-residue peptides. They also found that, in the presence of small unilamellar palmitoyloleoyl phosphatidylcholine (POPC) vesicles, the wild-type but not the N-terminal mutant peptide adopts a predominantly α-helical conformation at low pH. Burger et al. (1991) found that while the wild-type peptide penetrates dioleoyl PC/dioleoyl phosphatidylethanolamine (PE)/cholesterol monolayers more effectively than the mutant, both peptides have similar α-helical content and suggested that the formation of an α-helix is not sufficient for fusion activity. HA2 peptides with 20 amino acids have been shown to induce lipid mixing between small unilamellar vesicles composed of POPC, again at neutral pH, whereas the mutant peptides are ineffective (Wharton et al., 1988). Similar peptides corresponding to sequences from the A/PR/8/34 and B/Lee/40 strains of influenza virus also mediate lipid mixing between sonicated egg PC or POPC vesicles, respectively, although the former peptide requires acidic pH (Lear & De Grado, 1987; Murata et al., 1987). Since small vesicles may have phospholipid packing defects that render them more susceptible to fusion (Wilschut, Düzgüneş & Papahadjopoulos, 1981; Düzgüneş, 1985; Parente, Nir & Szoka, 1988; Stegmann et al., 1989), it is essential to investigate the fusion activity of such peptides using large unilamellar vesicles. It is also of interest to find the minimal peptide unit that is required for the induction of various effects on membranes, such as membrane aggregation, lipid mixing, fusion and destabilization. It was shown previously that while a pH-sensitive synthetic peptide of 30 amino acids is able to destabilize phospholipid vesicles, its fragments up to 16 amino acids do not cause any significant destabilization (Subbarao et al., 1987).

We investigated the membrane destabilizing and fusogenic activities of several N-terminal peptides from the fusion glycoproteins of influenza, Sendai, and vesicular stomatitis viruses (the Table). We monitored the destabilization of membranes by the release of 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and N,N′-*p*-xylylenebis (pyridinium bromide) (DPX) initially coencapsulated in large unilamellar liposomes. We monitored vesicle aggregation and membrane fusion by means of resonance energy transfer assays involving N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) PE (NBD-PE) and N-(lissamine) rhodamine B sulfonyl PE (Rh-PE) incorporated into the liposome membrane. We also monitored the intermixing of internal aqueous contents of vesicles as a measure of membrane fusion by encapsulating ANTS and DPX in separate populations of vesicles. A preliminary report of our findings has been presented earlier (Düzgüneş & Shavnin, 1988).

Materials and Methods

Materials

Bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC), Rh-PE and NBD-PE were provided by Avanti Polar Lipids (Alabaster, AL) and stored in chloroform under argon at −70°C. Cholesterol was obtained from Behring Diagnostics (La Jolla, CA) and recrystallized twice from methanol. *n*-Dodecyl octaethylene monoether ($C_{12}E_8$) was from Calbiochem (San Diego, CA). ANTS and DPX were obtained from Molecular Probes (Eugene,

OR). NaCl was from Fisher. EDTA, N-tris (hydroxymethyl)-methyl aminoethane sulfonic acid (TES) and 2(N-morpholino) ethane sulfonic acid (MES) were from Sigma (St. Louis, MO). Sodium citrate was from Mallinckrodt. Water was distilled twice, the second time in an all-glass apparatus, and purified further in a Barnstead Nanopure system.

Peptides

Peptides were synthesized either by Biosearch (San Rafael, CA) or the Biomolecular Resource Center, UCSF, using an Applied Biosystems 430A peptide synthesizer. The purity of the peptides was assessed to be more than 90% by HPLC analysis. Amino acid analysis indicated the expected composition. All peptides contained an additional cysteine at the C-terminus, both to keep this terminus constant between the peptides and for use in coupling the peptides to liposomes or a carrier. The peptides were very hydrophobic and hence would not dissolve in water. A large number of solvent systems were tested, and the best solvent was found to be 2 M guanidine hydrochloride/50% ethanol. The stock peptide solution was diluted 100-fold when injected into the buffer containing the phospholipid vesicles in the fluorometer cuvette. Control additions of the solvent or solvent containing a hydrophilic control peptide (a 17-amino acid consensus sequence form calcium-dependent membrane-binding proteins (Geisow et al., 1986)) to the vesicles produced no significant effects on the permeability or lipid mixing properties of the vesicles.

Preparation of Phospholipid Vesicles

Large unilamellar vesicles, or liposomes (LUV), were prepared by reverse-phase evaporation followed by extrusion four times through polycarbonate membranes (Nuclepore; Pleasanton, CA) of 100-nm pore diameter (Szoka et al., 1980; Düzgüneş et al., 1983). The size distribution of the vesicles was determined by dynamic light scattering in a Coulter N4 MD submicron particle analyzer. The mean diameter of the vesicles was about 150 nm.

Lipid Mixing Assays

Three populations of vesicles were prepared for the resonance energy transfer assay for lipid mixing ("probe dilution" assay; Struck, Hoekstra & Pagano, 1981; Rosenberg, Düzgüneş & Kayalar, 1983; Düzgüneş et al., 1985, 1987): Labeled vesicles, containing 1 mole % each of Rh-PE and NBD-PE, unlabeled vesicles, and vesicles to be used for the calibration of fluorescence to 100%, containing 0.2 mole % of each probe. The lipid concentration of the vesicle preparation was determined by phosphorus analysis (Bartlett, 1959). Labeled vesicles were mixed with unlabeled vesicles at a 1 : 4 ratio, at a total lipid concentration of 50 nmol/ml in 1 ml 100 mM NaCl, 5 mM TES, 5 mM MES, 5 mM citrate, pH 7.4 ("medium buffer") at 25°C. Complete mixing of the labeled and unlabeled vesicles would produce a "mega-vesicle" containing 0.2 mole % of each probe (Düzgüneş et al., 1985). Thus, the fluorescence intensity of vesicles (50 nmol lipid/ml) containing 0.2 mole % of each probe was set to 100% (designated F_{max}). The fluorescence intensity of the labeled vesicles was taken as 0%. Dilution of the probe molecules from labeled vesicles into unlabeled vesicles results in the decrease of the efficiency of energy transfer from NBD to Rh, and hence the fluorescence intensity of NBD increases. The probe dilution method has been shown to be insensitive to the mere aggregation of vesicles and to be a reliable indicator of lipid mixing during membrane fusion (Düzgüneş et al., 1987). Fluorescence measurements were made in an SLM 4000 fluorometer and recorded on an Omniscribe chart recorder. The excitation wavelength was 450 nm, and the emission wavelength was 520 nm with the monochromator slits set at 4 nm. The fluorometer cuvette was stirred continuously and thermostatted to 25°C.

For the "probe mixing" assay (Hoekstra, 1982; Düzgüneş et al., 1987) two populations of vesicles were prepared, one containing 1 mole % of NBD-PE and the other containing 1 mole % of Rh-PE. As well as monitoring lipid mixing between vesicles, this assay is a sensitive indicator of vesicle aggregation, since resonance energy transfer has a sharp dependence ($1/r^6$) on the distance between the probes.

Release of Aqueous Contents

The breakdown of the permeability barrier function of liposome membranes in the presence of peptides was determined by coencapsulating 12.5 mM ANTS and 45 mM DPX, with 10 mM TES, pH 7.4, adjusted to the osmolality of the medium buffer using NaCl (Ellens, Bentz & Szoka, 1984; Düzgüneş et al., 1985). After preparation of LUV as described above, unencapsulated material was separated from the liposomes by gel filtration on a Sephadex G-75 (Pharmacia, Piscataway, NJ) column using medium buffer for elution. The fluorescence scale was set to 100% (F_{max}) by lysing the vesicles with 0.8 mM (final concentration) $C_{12}E_8$. The residual fluorescence of the intact vesicles (which was very close to that of the buffer) was set to 0% fluorescence.

Aqueous Contents Mixing Assay for Membrane Fusion

Intermixing of aqueous contents during membrane fusion was monitored by the ANTS/DPX assay (Düzgüneş et al., 1985; Ellens, Bentz & Szoka, 1985), in which one population of vesicles contained 25 mM ANTS and the other 90 mM DPX, with 10 mM TES, pH 7.4, and adjusted to the osmolality of the medium buffer. Unencapsulated material was separated from the vesicles as described above. The two populations were mixed at a 1 : 1 ratio at a final lipid concentration of 50 nmol/ml. ANTS fluorescence was measured using a Corning 3-68 high-pass filter (>530 nm) with the excitation monochromator set at 360 nm. The initial fluorescence of the vesicle mixture was set at 100% (corresponding to 0% fusion). The fluorescence of vesicles containing coencapsulated ANTS/DPX was set to 0% (corresponding to 100% fusion, a hypothetical situation in which the contents of all the vesicles have intermixed).

Results

Peptides from Vesicular Stomatitis Virus G-Protein

The "probe mixing" assay, in which NBD-PE and Rh-PE are incorporated into separate populations of vesicles, monitors the combined effect of lipid mix-

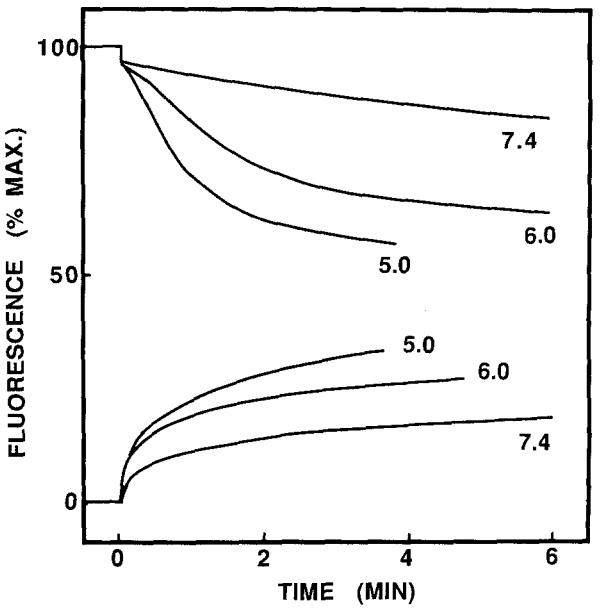

Fig. 1. The pH dependence of the effect of G.7 on probe mixing between and ANTS/DPX leakage from large unilamellar PS vesicles. The upper curves indicate the time course of the decrease of NBD-PE fluorescence due to resonance energy transfer to Rh-PE, the fluorophores being incorporated in separate populations of vesicles. The decrease could result from both aggregation and intermixing of membrane components. The lower curves show the increase of ANTS fluorescence due to dilution of the probe and its collisional quencher into the medium. Ten μl of a 0.667-mg/ml stock solution of the peptide was added to 1 ml of PS vesicles (50 nmol lipid/ml in 100 mM NaCl, 5 mM TES, 5 mM MES, 5 mM citrate, adjusted to the indicated pH) at a final concentration of 6.7 μg/ml. The temperature was 25°C.

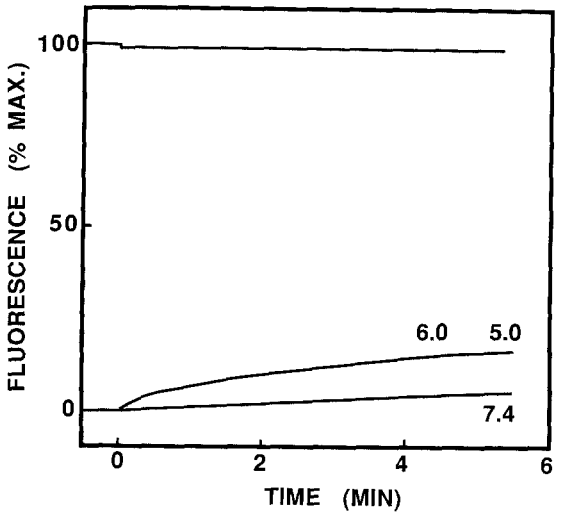

Fig. 2. The pH dependence of the effect of G.7 on probe dilution between and intermixing of aqueous contents of large unilamellar PS vesicles. The upper curve represents ANTS fluorescence at pH 7.4, 6.0 and 5.0. The lower curves show the time course of NBD-PE fluorescence, an increase indicating the dilution of both NBD-PE and Rh-PE from labeled to unlabeled vesicles, at the indicated pH. Other conditions were as in Fig. 1.

ing and aggregation of vesicles (Düzgüneş et al., 1987). Addition of the 7-amino-acid N-terminal peptide of the G-protein of vesicular stomatitis virus (G.7; the Table) to a suspension of PS vesicles caused the decrease of fluorescence due to resonance energy transfer from NBD to Rh (Fig. 1). The initial rate and extent of probe mixing increased as the pH was decreased. The concentration of the peptide was chosen to reveal significant differences under the different experimental conditions utilized in our studies, such as membrane composition and pH.

The effect of peptides on the permeability barrier properties of phospholipid membranes can be monitored by the release of encapsulated fluorophores (Düzgüneş & Gambale, 1988; Shiffer et al., 1988; Hoyt & Gierasch, 1991; Rafalski et al., 1991). The fluorescence of ANTS increases when the fluorophore is released into the medium where the collisional quencher DPX is no longer effective when it is diluted. The effect of G.7 on the release of ANTS/DPX from PS vesicles was also pH dependent (Fig. 1). The release was initially rapid but slowed down over time. To ascertain that leakage was not induced by the dimerization of G.7 via oxidation of the C-terminal cysteine sulfhydryls, the peptide was reduced by treatment with dithiothreitol; identical results were obtained as described above.

The "probe dilution" method has been shown to be insensitive to the mere aggregation of phospholipid vesicles and is therefore a more reliable indicator of lipid mixing between membranes than the "probe mixing" method (Düzgüneş et al., 1987). A low level of lipid mixing was observed at pH 7.4 in the presence of G.7. The initial rate and extent increased at pH 6.0, but no further increase was noted at pH 5.0 (Fig. 2). The change in fluorescence intensity at low pH was considerably lower than that observed in the probe mixing assay.

Lipid mixing between interacting phospholipid vesicles may result from lipid transfer, mixing of outer monolayers, as well as membrane fusion (Düzgüneş, 1985; Düzgüneş & Bentz, 1988). The latter process is defined in our studies as the mixing of aqueous contents of vesicles. The ANTS/DPX assay for the intermixing of aqueous contents during membrane fusion indicated no change in ANTS fluorescence in the presence of G.7 at pH 7.4–5.0 (Fig. 2). This effect may be the result of either the rapid leakage of contents from vesicles interacting with

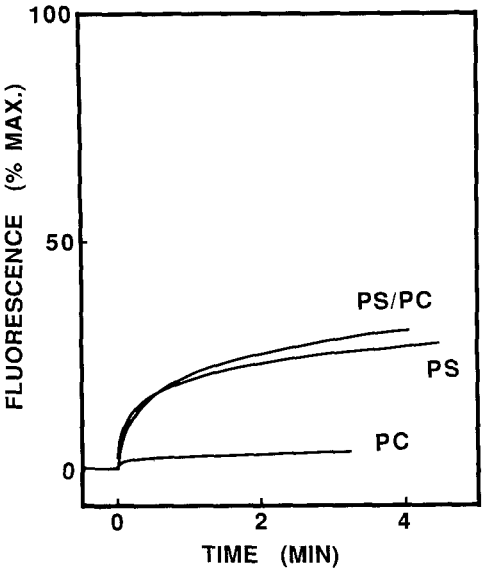

Fig. 3. Kinetics of G.7-induced release of encapsulated ANTS/DPX from large unilamellar vesicles composed of pure PC, PC/PS (1 : 1) or pure PS. The pH was 6.0. Other conditions were as in Fig. 1.

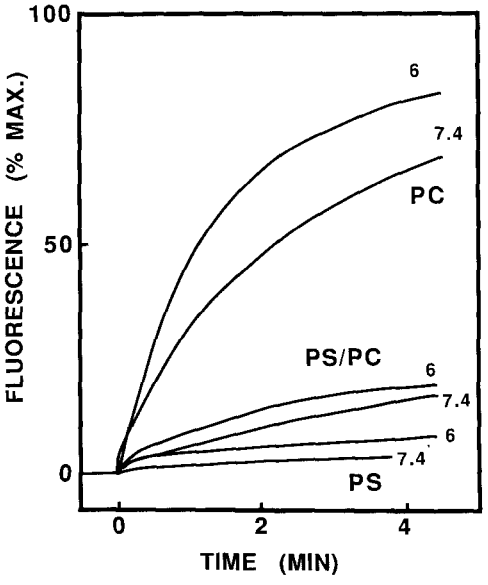

Fig. 4. Influence of lipid composition on the kinetics of HA2.17-induced release of ANTS/DPX from large unilamellar vesicles. The final peptide concentration was 6.7 μg/ml. For each lipid composition, the lower curve was obtained at pH 7.4 and the upper curve at pH 6.0.

G.7 or the absence of any coalescence of the internal aqueous space of the aggregated vesicles, despite lipid transfer between the vesicles. At this stage, we cannot rule out the possibility that the rapid leakage of contents may have prevented the ANTS and DPX from interacting during a relatively slow process of intermixing of internal aqueous spaces of the vesicles. However, this assay can observe membrane fusion even in liposome systems that exhibit rapid release, albeit transiently (Düzgüneş et al., 1985), and thus, any mixing of aqueous contents that occurs faster than the release of contents should have been observed.

The interaction of G.7 with membranes, as measured by the leakage of ANTS/DPX, was dependent on the phospholipid composition of the vesicles. PS-containing vesicles displayed rapid leakage in the presence of the peptide (Fig. 3), while vesicles composed of pure PC did not release their contents to a significant degree. The observation that not all the internal contents of the vesicles leaked as a result of peptide-membrane interaction may be attributed to the possibility that several peptides need to form a complex before the membrane is destabilized and that this is a dynamic process (Düzgüneş & Gambale, 1988; Parente, Nir & Szoka, 1990). The 11-amino acid peptide G.11 did not have a significant effect on the permeability of PS or PC vesicles and did not induce any lipid mixing between these vesicles (*data not shown;* the Table).

Peptides from Influenza Virus Hemagglutinin (HA2)

The membrane action of the N-terminal peptides of the cleaved hemagglutinin of influenza virus, HA2.7, HA2.10, HA2.17 (the Table), had different effects on the release of aqueous contents from PC vesicles. The most significant effect was observed with HA2.17 (Fig. 4). The extent of release 1 min after the addition of the peptide to PC vesicles at pH 7.4 was 28% F_{max} for HA2.17, while for HA2.10 and HA2.7 these values were 4.2% F_{max} and 3.1% F_{max}, respectively (*data not shown*). The effect of HA2.17 was enhanced when the pH was lowered to 6.0. PC vesicles were the most sensitive to the effect of HA2.17, compared to PS/PC and pure PS vesicles. The interaction of HA2.17 with each of these vesicles was enhanced at pH 6.0.

The hemagglutinin peptides did not cause any appreciable aggregation or lipid mixing in PC or PS vesicle suspensions at pH 7.4–5.0 (*data not shown*). At pH 5.0, addition of 13.4 μg/ml HA2.17 to PC vesicles caused an increase of about 2% F_{max} within 15 sec, in the probe dilution assay. This effect was not observed when the peptide concentration was reduced to 6.7 μg/ml or when the pH was increased to 6.0 or 7.4. In some experiments the aggregation of negatively charged vesicles was mediated by adding small nonfusogenic concentrations of calcium or

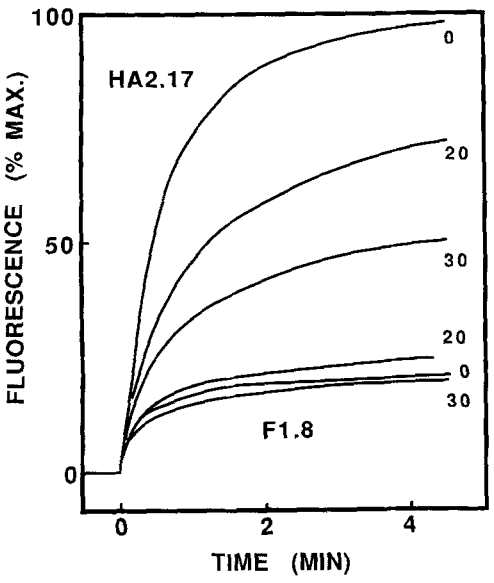

Fig. 5. The effect of the cholesterol content of PC vesicles on the kinetics of ANTS/DPX release from large unilamellar vesicles induced by F1.8 or HA2.17. The cholesterol mole % is given next to each curve. The pH was 7.4, and the peptide concentration was 6.7 μg/ml. Other conditions were as in Fig. 1.

magnesium; the peptides were unable to induce fusion even under these conditions (*data not shown*).

Peptides of Sendai Virus F1-Protein

The 8-amino acid peptide, F1.8, corresponding to the N-terminus of the Sendai virus F1-protein has the highest hydrophobicity index per amino acid (Eisenberg et al., 1984) among the peptides used in our studies (the Table). However, the kinetics and extent of ANTS/DPX leakage induced by F1.8 in PC liposomes was considerably lower than that produced by HA2.17 (Fig. 5). Lowering the pH to 6.0 enhanced the extent of leakage from PC liposomes by 50%, and the inclusion of PS in PC liposomes (1 : 1) reduced the leakage induced by this peptide by 88%. No lipid mixing was observed with PC or PS vesicles between pH 5.0 and 7.4 (*data not shown*).

The presence of cholesterol in the target membrane can influence virus fusion in some systems (Düzgüneş, 1988; Hoekstra, 1990). Cholesterol has been proposed to facilitate the insertion of the N-terminus of Sendai F1 into the membrane, thought to be accompanied by the formation of a stereospecific complex between the two molecules (Asano & Asano, 1985). It was therefore of interest to examine the effect of cholesterol on the interaction of F1.8 with phospholipid vesicles. The differences in the kinetics of leakage, between vesicles containing 0, 20 or 30 mole % cholesterol in PC vesicles, were not significant (Fig. 5). In contrast, the inclusion of cholesterol in PC vesicles affected the HA2.17-induced destabilization. Both the initial rate and extent of release were decreased when the cholesterol content of PC vesicles was increased from 0 to 30 mole % (Fig. 5).

Discussion

Our results indicate that the N-terminal segments of envelope glycoproteins of several lipid enveloped viruses can interact with target membranes and destabilize them. In the case of HA2, G- and F1-proteins, this interaction is specific for the phospholipid composition of the target membranes and is enhanced at low pH. Our observations support the hypothesis that these viral envelope proteins mediate fusion by inserting their N-termini into the membrane with which the virus is fusing. Independent evidence for the insertion of the HA2 N-terminus has been obtained by hydrophobic photolabeling (Harter et al., 1989). The mechanism by which peptide insertion causes the fusion of the viral and target membranes is not known. It is possible that the destabilization increases the hydrophobicity of the membrane surface (Ohki & Düzgüneş, 1979; Ohki, 1988) and changes the water structure of the region of adhesion between the viral and target membranes. The increased hydrophobicity may alter locally the intermembrane interaction energy, possibly forcing the viral envelope bilayer to adapt to this new microenvironment by "out-of-plane" molecular motions of the phospholipids, as proposed by Leikin et al. (1987) for thermal fluctuations. An alternate possibility is that the exposed N-terminus also interacts with the viral membrane (Ruigrok et al., 1986), thereby creating a hydrophobic interface which then interacts with the hydrophobic region on the target membrane. It is possible that one monomer of the HA trimer interacts with the target membrane while another interacts with the virus membrane.

The induction of nonbilayer lipid structures by a hydrophobic 19-amino acid peptide corresponding to the N-terminus of measles virus F1-protein suggests the possibility that membrane fusion is mediated via the formation of such structures (Yeagle et al., 1991). However, the correlation of membrane fusion and the formation of these structures is not absolute (Yeagle et al., 1991); in addition, hydrophobic "signal peptides" also induce nonlamellar structures in phospholipid membranes (Killian et al., 1990). Studies with mutant peptides have shown correlations between the ability of the peptides to insert into membranes and the capacity of the parent pro-

tein to mediate either fusion, as in the case of influenza HA (Gething et al., 1986; Düzgüneş & Gambale, 1988; Wharton et al., 1988; Rafalski et al., 1991), or membrane translocation, as in the *E. coli* outer membrane protein OmpA (Hoyt & Gierasch, 1991). These observations suggest that the ultimate function of "fusion" or "signal" peptides depends on the remainder of the protein.

Peptide-induced lipid mixing and leakage were both increased by decreasing pH. This may be attributed to various factors including conformational changes of the peptide, alteration of the charge on the peptide thereby reducing the free energy of transfer of the peptide from the aqueous phase to the lipid bilayer (Ohnishi, 1988), and, in the case of PS, reduction of the magnitude of the membrane surface potential. Since the effects of the peptides *are* observed at pH 7.4, however, low pH does not appear to be *required* for membrane destabilization. This proposal is supported by the negative free energy of transfer of the F1 and HA2 N-termini from the aqueous to the lipid bilayer phase even at neutral pH (Ohnishi, 1988). Thus, the conformational change of the HA2 induced by low pH would expose the N-terminus, which would then insert into the target membrane in a pH-independent manner. Since low pH-induced fusion of influenza virus with erythrocyte ghosts is arrested when the pH is returned to neutral (Stegmann et al., 1986), while the low pH-induced conformational change of HA and binding of the ectodomain of HA to liposomes is thought to be irreversible (Skehel et al., 1982; Sato, Kawasaki & Ohnishi, 1983; Doms, Helenius & White, 1985), it is intriguing that fusion does not proceed at neutral pH once it has been initiated. We have proposed earlier that the process of conformational change induced by low pH may be necessary for fusion (Düzgüneş & Gambale, 1988). The recent proposal by Puri et al. (1990) that fusion occurs during the transition from a "relaxed" conformation at low pH to an "active" conformation of HA while the molecule is associated with the target membrane also supports our hypothesis. It is also possible that the low pH conformations of other sections of the HA are necessary for fusion, as also suggested by Wharton et al. (1988), particularly since the molecule appears to constitute a formidable steric hindrance to the approach of the N-terminus to the target membrane and to the apposition of the viral and target membranes, even after the folding of the globular heads (*see* White & Wilson, 1987).

The specificity of G.7 for PS over PC membranes may be related to the proposal that PS may be the cellular receptor for VSV (Schlegel et al., 1983), in that the N-terminus of the G-protein may bind to PS molecules exposed on the cell surface. Arguing against the involvement of the N-terminus of G-protein in fusion is the observation by Woodget and Rose (1986) that the mutation of the N-terminal lysine to glutamic acid does not affect the cell-cell fusion activity of the G-protein expressed in cultured cells. However, the substitution of one polar amino acid for another does not alter appreciably the overall hydrophobicity of this region and, thus, may not be a critical mutation. As long as the hydrophobicity is maintained, a specific sequence may not be necessary for fusion.

In contrast, HA2.17 interacts preferentially with PC membranes. Since G.7 and HA2.17 have the same hydrophobicities per unit (the Table) but have different effects on membranes of the same composition, the specific amino acid sequence or composition must play a role in their specific actions on membranes. The shorter HA2 peptides, HA2.7 and HA2.10, with average hydrophobicities of 0.79 and 0.78, respectively, were considerably less effective than HA2.17. Thus, the length of the peptides appears to have a distinct effect on their interaction with phospholipid membranes, possibly because of the differences in the allowable conformations of the peptides. A similar observation was made by Lear and DeGrado (1987) with respect to the interaction of HA2 peptides from the B/Lee/40 strain of influenza virus with sonicated POPC vesicles, in that the 20-amino acid, but not the 16-amino acid peptide, adopted a helical conformation when bound to vesicles. The inefficiency of the longer G peptide (G.11) to destabilize membranes may be related not only to the lower hydrophobicity of this peptide (the Table) but also to the allowable conformations for this length of peptide with this sequence of amino acids.

F1.8 destabilizes PC membranes to a greater extent than PS or PS/PC membranes. This observation, however, does not imply that the viral F1-protein as a whole interacts less efficiently with negatively charged membranes. The rate of fusion of Sendai virus with cardiolipin/PC liposomes is inhibited as the mole fraction of PC is increased (Klappe et al., 1986), and F1 has been shown to penetrate into cardiolipin or PS membranes (Novick & Hoekstra, 1988). Sendai virus induces leakage from small unilamellar vesicles composed of PS and not from those of PC (Amselem et al., 1985). Nevertheless, Citovsky, Blumenthal and Loyter (1985) have suggested that fusion of the virus with PC/cholesterol liposomes, and not negatively charged liposomes, reflects the properties of the virus involved in fusion with cellular membranes. The observation that the destabilization is enhanced at lower pH correlates with the enhanced fusion of Sendai virus with various liposomes at reduced pH (Klappe et al., 1986; Nir, Klappe & Hoekstra, 1986). The rate of fusion

of an isolate of Sendai virus with cultured cells is also enhanced at mildly acidic pH (Pedroso de Lima et al., 1991). We should point out, however, that the virus-cell membrane fusion reaction is considerably more complex than the insertion of the hydrophobic segment of the envelope protein and may have different molecular requirements.

The presence of cholesterol decreases the effect of HA2.17 on PC vesicles, but it does not affect the action of F1.8. This observation suggests that the initial interaction of the N-terminus of the Sendai F-protein with target membranes may not require the presence of cholesterol, in contrast to an earlier proposal (Asano & Asano, 1985). It is possible that cholesterol mediates the conformational changes of the F-protein subsequent to insertion of the N-terminus into the target membrane.

The peptides investigated in this study do not induce observable membrane fusion in large unilamellar phospholipid vesicles. Although G.7 causes probe dilution in PS vesicles, this is not accompanied by the intermixing of aqueous contents. Probe dilution may have resulted from peptide-mediated lipid exchange or the intermixing of the outer monolayers of labeled and unlabeled vesicles, a process that does not necessarily lead to aqueous contents mixing (Rosenberg et al., 1983; Düzgüneş & Bentz, 1988). This observation emphasizes the need to monitor fusion by independent techniques. The other peptides tested do not even mediate aggregation, as monitored by the probe mixing assay and by 90° light scattering.

All studies on the fusion activity of influenza virus peptides reported so far have utilized small unilamellar vesicles prepared by sonication (Lear & DeGrado, 1987; Murata et al., 1987; Wharton et al., 1988). Such vesicles, because of their inherent lipid packing defects, may be prone to fusion under conditions which do not cause the fusion of large unilamellar vesicles (Wilschut et al., 1981; Parente et al., 1988; Stegmann et al., 1989; M. Murata and S.I. Ohnishi, *personal communication*). Our studies indicate that large vesicles do not undergo significant fusion in the presence of the various viral peptides we have utilized. A 20-amino acid peptide from the X-31 HA2 N-terminus was also shown not to induce the fusion of large POPC vesicles (*unpublished data* of Wilschut et al. cited in Rafalski et al., 1991). It will be of interest to examine the effect on membrane destabilization and fusion of longer peptides which may adopt conformations closer to those of the native N-terminal region. We should also note, however, that the fusion induced by such peptides may not necessarily reflect the fusogenic activity of viral fusion proteins. Although the N-termini of the membrane-spanning subunits of influenza and human immunodeficiency virus envelope glycoproteins are clearly involved in the fusion of these viruses, as determined by studies utilizing site-directed mutagenesis (Gething et al., 1986; Kowalski et al., 1987), the remainder of the protein and the other members of the oligomeric structure formed by the proteins must also participate in the fusion process (*see* discussion by Hoekstra (1990)). Studies on the molecular and structural requirements for the membrane-destabilizing effects of the N-terminal peptides may be relevant to understanding how these segments of the viral envelope proteins mediate membrane fusion. In addition, examination of the structural requirements of model peptides for interaction with phospholipid membranes (Subbarao et al., 1987; Parente et al., 1988, 1990; Suenaga et al., 1989; Burger et al., 1991; Hoyt & Gierasch, 1991) may provide additional insights into the mechanisms of the initial steps involved in viral protein-mediated membrane fusion.

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References

Amselem, S., Barenholz, Y., Loyter, A., Nir, S., Lichtenberg, D. 1986. Fusion of Sendai virus with negatively charged liposomes as studied by pyrene-labelled phospholipid liposomes. *Biochim. Biophys. Acta* **860**:301–313

Asano, K., Asano, A. 1985. Why is a specific amino acid sequence of F glycoprotein required for the membrane fusion reaction between envelope of HVJ (Sendai virus) and target cell membranes? *Biochem. Int.* **10**:115–122

Asano, K., Murachi, T., Asano, A. 1983. Structural requirements for hemolytic activity of F-glycoprotein of HVJ (Sendai virus) studied by proteolytic digestion. *J. Biochem.* **93**:733–741

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466–468

Burger, K.N.J., Wharton, S.A., Demel, R.A., Verkleij, A.J. 1991. The interaction of synthetic analogs of the N-terminal fusion sequence of influenza virus with a lipid monolayer. Comparison of fusion-active and fusion-defective analogs. *Biochim. Biophys. Acta* **1065**:121–129

Chernomordik, L.V., Melikyan, G.B., Chizmadzhev, Y.A. 1987. Biomembrane fusion: A new concept derived from model studies using two interacting planar lipid bilayers. *Biochim. Biophys. Acta* **906**:309–352

Citovsky, V., Blumenthal, R., Loyter, A. 1985. Fusion of Sendai virions with phosphatidylcholine-cholesterol liposomes re-

flects the viral activity required for fusion with biological membranes. *FEBS Lett.* **193:**135–140

Doms, R.W., Helenius, A., White, J. 1985. Membrane fusion activity of the influenza hemagglutinin. The low pH-induced conformational change. *J. Biol. Chem.* **260:**2973–2981

Düzgüneş, N. 1985. Membrane fusion. *In:* Subcellular Biochemistry. D.B. Roodyn, editor. Vol. 11, pp. 195–286. Plenum, London

Düzgüneş, N., 1988. Cholesterol and membrane fusion. *In:* Biology of Cholesterol. P.L. Yeagle, editor, pp. 197–212. CRC Press, Boca Raton (FL)

Düzgüneş, N., Allen, T.M., Fedor, J., Papahadjopoulos, D. 1987. Lipid mixing during membrane aggregation and fusion. Why fusion assays disagree. *Biochemistry* **26:**8435–8442

Düzgüneş, N., Bentz, J. 1988. Fluorescence assays for membrane fusion. *In:* Spectroscopic Membrane Probes. L.M. Loew, editor. pp. 117–159. CRC Press, Boca Raton (FL)

Düzgüneş, N., Gambale, F. 1988. Membrane action of synthetic peptides from influenza virus hemagglutinin and its mutants. *FEBS Lett.* **227:**110–114

Düzgüneş, N., Shavnin, S.A. 1988. Influence of synthetic peptides derived from viral envelope proteins on the permeability and fusion of phospholipid vesicles. *Abstr. Frumkin Symposium on Bioelectrochemistry,* Suzdal, USSR

Düzgüneş, N., Straubinger, R.M., Baldwin, P.A., Friend, D.S., Papahadjopoulos, D. 1985. Proton-induced fusion of oleic acid/phosphatidylethanolamine liposomes. *Biochemistry* **24:**3091–3098

Düzgüneş, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D.S., James, T.L., Papahadjopoulos, D. 1983. Physicochemical characterization of large unilamellar vesicles prepared by reverse-phase evaporation. *Biochim. Biophys. Acta* **732:**289–299

Eisenberg, D., Schwarz, E., Komoramy, M., Wall, R. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **179:**125–142

Ellens, H., Bentz, J., Szoka, F.C. 1984. pH-induced destabilization of phosphatidylethanolamine-containing liposomes. *Biochemistry* **23:**1532–1538

Ellens, H., Bentz, J., Szoka, F.C. 1985. H^+ and Ca^{2+}-induced fusion and destabilization of liposomes. *Biochemistry* **24:**3099–3106

Geisow, M.J., Fritsche, V., Hexham, J.M., Dash, B., Johnson, T. 1986. A consensus amino-acid sequence repeat in *Torpedo* and mammalian Ca^{2+}-dependent membrane-binding proteins. *Nature* **320:**636–638

Gething, M.-J., Doms, R.W., York, D., White, J.M. 1986. Studies on the mechanism of membrane fusion: Site-specific mutagenesis of the hemagglutinin of influenza virus. *J. Cell Biol.* **102:**11–23

Gething, M.J., White, J.M., Waterfield, M.D. 1978. Purification of the fusion protein of Sendai virus: Analysis of the NH_2-terminal sequence generated during precursor activation. *Proc. Natl. Acad. Sci. USA* **75:**2737–2740

Harter, C., James, P., Bächi, T., Semenza, G., Brunner, J. 1989. Hydrophobic binding of the ectodomain of influenza hemagglutinin to membranes occurs through the "fusion peptide." *J. Biol. Chem.* **264:**6459–6464

Hoekstra, D. 1982. Kinetics of intermixing of lipids and mixing of aqueous contents during vesicle fusion. *Biochim. Biophys. Acta* **692:**171–175

Hoekstra, D. 1990. Membrane fusion of enveloped viruses: Especially a matter of proteins. *J. Bioenerg. Biomembr.* **22:**121–156

Hoekstra, D., Kok, J.W. 1989. Entry mechanisms of enveloped viruses. Implications for fusion of intracellular membranes. *Biosci. Rep.* **9:**273–305

Hoekstra, D., Wilschut, J. 1989. Membrane fusion of artificial and biological membranes: Role of local membrane dehydration. *In:* Water Transport in Biological Membranes. G. Benga, editor. pp. 143–176. CRC Press, Boca Raton (FL)

Hoyt, D.W., Gierasch, L.M. 1991. Hydrophobic content and lipid interactions of wild-type and mutant OmpA signal peptides correlate with their in vivo function. *Biochemistry* **30:**10155–10163

Hsu, M.C., Scheid, A., Choppin, P.W. 1981. Activation of the Sendai virus fusion protein (F) involves a conformational change with exposure of a new hydrophobic region. *J. Biol. Chem.* **256:**3557–3563

Kelsey, D.R., Flanagan, T.D., Young, J., Yeagle, P.L. 1990. Peptide inhibitors of enveloped virus infection inhibit phospholipid vesicle fusion and Sendai virus fusion with phospholipid vesicles. *J. Biol. Chem.* **265:**12178–12183

Killian, J.A., de Jong, A.M.P., Bijvelt, J., Verkleij, A.J., de Kruijff, B. 1990. Induction of non-bilayer lipid structures by functional signal peptides. *EMBO J.* **9:**815–819

Klappe, K., Wilschut, J., Nir, S., Hoekstra, D. 1986. Parameters affecting fusion between Sendai virus and liposomes. Role of viral proteins liposome composition and pH. *Biochemistry* **25:**8252–8260

Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W.C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W., Sodroski, J. 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* **237:**1351–1355

Lear, J.D., De Grado, W.F. 1987. Membrane binding and conformational properties of peptides representing the NH_2 terminus of influenza virus. *J. Biol. Chem.* **262:**6500–6505

Leikin, S.L., Kozlov, M.M., Chernomordik, L.V., Markin, V.S., Chizmadzhev, Y.A. 1987. Membrane fusion: Overcoming the hydration barrier and local restructuring. *J. Theor. Biol.* **129:**411–425

Marsh, M., Helenius, A. 1989. Virus entry into animal cells. *Adv. Virus Res.* **36:**107–151

Murata, M., Sugahara, Y., Takahashi, S., Ohnishi, S.-I. 1987. pH-Dependent membrane fusion activity of a synthetic twenty amino acid peptide with the same sequence as that of the hydrophobic segment of influenza virus hemagglutinin. *J. Biochem.* **102:**957–962

Nir, S., Klappe, K., Hoekstra, D. 1986. Mass action analysis of kinetics and extent of fusion between Sendai virus and liposomes. *Biochemistry* **25:**8261–8266

Norrby, E. 1971. The effect of a carbobenzoxy tripeptide on the biological activities of measles virus. *Virology* **44:**599–608

Novick, S.L., Hoekstra, D. 1988. Membrane penetration of Sendai virus glycoproteins during the early stages of fusion with liposomes as determined by hydrophobic photoaffinity labeling. *Proc. Natl. Acad. Sci. USA* **85:**7433–7437

Ohki, S. 1988. Surface tension, hydration energy and membrane fusion. *In:* Molecular Mechanisms of Membrane Fusion. S. Ohki, D. Doyle, T.D. Flanagan, S.K. Hui, and E. Mayhew, editors. pp. 123–138. Plenum, New York

Ohki, S., Düzgüneş, N. 1979. Divalent cation-induced interaction of phospholipid vesicles and monolayer membranes. *Biochim. Biophys. Acta* **552:**438–449

Ohnishi, S. 1988. Fusion of viral envelopes with cellular membranes. *In:* Membrane Fusion in Fertilization, Cellular Transport and Viral Infection. N. Düzgüneş and F. Bronner, editors. pp. 257–296. Academic, New York

Okada, Y. 1988. Sendai virus-mediated cell fusion. *In:* Membrane Fusion in Fertilization, Cellular Transport and Viral Infection. N. Düzgüneş and F. Bronner, editors. pp. 297–336. Academic, New York

Parente, R.A., Nir, S., Szoka, F.C., Jr. 1988. pH-dependent fusion of phosphatidylcholine small vesicles. *J. Biol. Chem.* **263:**4724–4730

Parente, R.A., Nir, S., Szoka, F.C., Jr. 1990. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry* **29:**8720–8728

Pedroso de Lima, M.C., Nir, S., Flasher, D., Klappe, K., Hoekstra, D., Düzgüneş, N. 1991. Fusion of Sendai virus with human HL-60 and CEM cells: Different kinetics of fusion for two isolates. *Biochim. Biophys. Acta* **1070:**446–454

Puri, A., Booy, F.P., Doms, R.W., White, J.M., Blumenthal, R. 1990. Conformational changes and fusion activity of influenza virus hemagglutinin of the H2 and H3 subtypes: Effects of acid pretreatment. *J. Virol.* **64:**3824–3832

Rafalski, M., Ortiz, A., Rockwell, A., van Ginkel, L.C., Lear, J.D., DeGrado, W.F., Wilschut, J. 1991. Membrane fusion activity of the influenza virus hemagglutinin: Interaction of HA2 N-terminal peptides with phospholipid vesicles. *Biochemistry* **30:**10211–10220

Richardson, C.D., Scheid, A., Choppin, P.W. 1980. Specific inhibition of paramyxovirus and myxovirus replication by oligopeptides with amino acid sequences similar to those at the N-termini of the F_1 or HA_2 viral polypeptides. *Virology* **105:**205–222

Rosenberg, J., Düzgüneş, N., Kayalar, C. 1983. Comparison of two liposome fusion assays monitoring the intermixing of aqueous contents and of membrane components. *Biochim. Biophys. Acta* **735:**173–180

Ruigrok, R.W.H., Wrigley, N.G., Calder, L.J., Cusak, S., Wharton, S.A., Brown, E.B., Skehel, J.J. 1986. Electron microscopy of the low pH structure of influenza virus hemagglutinin. *EMBO J.* **5:**41–49

Sato, S.B., Kawasaki, K., Ohnishi, S.-I. 1983. Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments. *Proc. Natl. Acad. Sci. USA* **80:**3153–3157

Schlegel, R., Tralka, S.T., Willingham, M.C., Pastan, I. 1983. Inhibition of VSV binding and infectivity by phosphatidylserine: Is phosphatidylserine a VSV binding site? *Cell* **32:**639–646

Schlegel, R., Wade, M. 1984*a*. Biologically active peptides of the vesicular stomatitis virus glycoprotein. *J. Virol.* **53:**319–323

Schlegel, R., Wade, M. 1984*b*. A synthetic peptide corresponding to the NH_2 terminus of vesicular stomatitis virus glycoprotein is a pH-dependent hemolysin. *J. Biol. Chem.* **259:**4691–4694

Shiffer, K., Hawgood, S., Düzgüneş, N., Goerke, J. 1988. The interactions of the low molecular weight group of surfactant-associated proteins (SP 5-18) with pulmonary surfactant lipids. *Biochemistry* **27:**2689–2695

Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A., Wiley, D.C. 1982. Changes in the conformation of influenza hemagglutinin at the pH optimum of virus mediated membrane fusion. *Proc. Natl. Acad. Sci. USA* **79:**968–972

Spear, P.G. 1987. Virus-induced cell fusion. *In:* Cell Fusion. A.E. Sowers, editor. pp. 3–32. Plenum, New York

Stegmann, T., Doms, R.W., Helenius, A. 1989. Protein-mediated membrane fusion. *Annu. Rev. Biophys. Chem.* **18:**187–211

Stegmann, T., Hoekstra, D., Scherphof, G., Wilschut, J. 1986. Fusion activity of influenza virus. A comparison between biological and artificial target membrane vesicles. *J. Biol. Chem.* **261:**10966–10969

Struck, D.K., Hoekstra, D., Pagano, R.E. 1981. Use of resonance energy transfer to monitor fusion. *Biochemistry* **20:**4093–4099

Subbarao, N.K., Parente, R.A., Szoka, F.C., Jr., Nadasdi, L., Pongracz, K. 1987. pH-dependent bilayer destabilization by an amphipathic peptide. *Biochemistry* **26:**2964–2972

Suenaga, M., Lee, S., Park, N.G., Aoyagi, H., Kato, T., Umeda, A., Amako, K. 1989. Basic amphipathic helical peptides induce destabilization and fusion of acidic and neutral liposomes. *Biochim. Biophys. Acta* **981:**143–150

Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., Papahadjopoulos, D. 1980. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. *Biochim. Biophys. Acta* **601:**559–571

Wharton, S.A., Martin, S.R., Ruigrok, R.W.H., Skehel, J.J., Wiley, D.C. 1988. Membrane fusion by peptide analogues of influenza virus hemagglutinin. *J. Gen. Virol.* **69:**1847–1857

White, J., Kielian, M., Helenius, A. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* **16:**151–195

White, J.M., Wilson, I.A. 1987. Anti-peptide antibodies detect steps in a protein conformational change: Low pH activation of the influenza virus hemagglutinin. *J. Cell Biol.* **105:**2887–2896

Wilschut, J., Düzgüneş, N., Papahadjopoulos, D. 1981. Calcium/magnesium specificity in membrane fusion: Kinetics of aggregation and fusion of phosphatidylserine vesicles and the role of bilayer curvature. *Biochemistry* **20:**3126–3133

Woodget, C., Rose, J.K. 1986. Amino-terminal mutation of the vesicular stomatitis virus glycoprotein does not affect its fusion activity. *J. Virol.* **59:**486–489

Yeagle, P.L., Epand, R.M., Richardson, C.D., Flanagan, T.D. 1991. Effects of the 'fusion peptide' from measles virus on the structure of N-methyl dioleoylphosphatidyl-ethanolamine membranes and their fusion with Sendai virus. *Biochim. Biophys. Acta* **1065:**49–53

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