Membrane Fusion Activity of the Influenza Virus Hemagglutinin: Interaction of HA2 N-Terminal Peptides with Phospholipid Vesicles†

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ABSTRACT: We have investigated the interaction of a number of synthetic 20-residue peptides, corresponding to the HA2 N-terminus of the influenza virus hemagglutinin (X31 strain), with phospholipid vesicles and monolayers. Besides the wild-type sequence, two peptides were studied with mutations corresponding to those previously studied in entire HA's expressed in transfected cells [Gething et al., (1986) *J. Cell. Biol. 102*, 11–23]. These mutations comprised a single Glu replacement for Gly at the N-terminus ("E1" mutant) or at position 4 ("E4") of the HA2 subunit and were shown to produce striking alterations in virus-induced hemolysis and syncytia formation, especially for E1. The X31 "wild-type" (wt) peptide and its E4 variant are shown here to have the capacity to insert into phosphatidylcholine (POPC) large unilamellar vesicle (LUV) membranes in a strictly pH-dependent manner, penetration being marginal at pH 7.4 and significant at pH 5.0. Bilayer insertion was evident from a shift in the intrinsic Trp fluorescence of the wt and E4 peptides and from the induction of calcein leakage from POPC LUV and correlated well with the peptides' ability at pH 5.0 to penetrate into POPC monolayers at initial surface pressures higher than 30 mN/m. By contrast, the E1 peptide was found, at pH 5.0, to bind less tightly to vesicles (assessed by a physical separation method) and to cause much less leakage of POPC LUV than the wt, even under conditions where the peptides were bound to approximately the same extent. Consistent with the correlation between leakage and penetration observed for the wt peptide at pH 5 versus 7, the E1 peptide, even at low pH, showed much less lipid-vesicle-induced shift of its Trp fluorescence than wt, caused a much slower rate of leakage of vesicle contents, and did not insert into POPC monolayers at surface pressures beyond 28.5 mN/m. Circular dichroism spectroscopy measurements of peptides in POPC SUV showed that the conformations of all three peptides are sensitive to pH, but only the wt and E4 peptides became predominantly α-helical at acid pH.

Hemagglutinin (HA),[1] the major spike glycoprotein of influenza virus, plays a crucial role in the infectious entry of the virus into its host cell. First, HA mediates the initial attachment of the virus particles to sialic acid containing receptors localized in coated-pit regions on the cell surface (Rogers et al., 1983; Wiley & Skehel, 1987; Sauter et al., 1989). Virus attachment is followed by internalization of intact virus particles through receptor-mediated endocytosis, directing the virions to the endosomal cell compartment (Matlin et al., 1981; Yoshimura & Ohnishi, 1984; Richman et al., 1986; Stegmann et al., 1987a). The mildly acidic pH in the lumen of the endosomes (Mellman et al., 1986) then triggers a fusion reaction between the viral envelope and the limiting membrane of the endosomes, through which the viral nucleoscapsid gains access to the cellular cytoplasm. This low pH induced fusion process is mediated by the viral HA as well (White et al., 1982, 1983; White, 1990; Stegmann et al., 1989; Doms et al., 1990).

HA is the best characterized membrane fusion protein so far [for reviews, see Doms et al. (1990), White (1990), and Wiley and Skehel (1987)]. The HA spike is a homotrimer, each monomer consisting of two disulfide-linked subunits, HA1 and HA2, which are generated from a single polypeptide chain, HA0, by posttranslational cleavage involving a host cell protease (Klenk et al., 1975; Orlich et al., 1990). HA1 contains the sialic acid binding pocket (Sauter et al., 1989). The N-terminus of HA2, generated by the activating cleavage, appears crucial for the expression of fusion activity of HA: uncleaved HA0 is not fusion active (Klenk et al., 1975; Sato et al., 1983; White et al., 1982, 1983; White, 1990), whereas site-specific mutations within this region of the molecule severely affect the fusion activity of HA (Gething et al., 1986). The N-terminus of HA2, the so-called "fusion segment", is a conserved stretch of some 20 amino acid residues that are mostly hydrophobic in nature (White, 1990). From the X-ray crystal structure of the bromelain-solubilized ectodomain of HA (BHA) (Wilson et al., 1981; Wiley & Skehel, 1987), it appears that at neutral pH the fusion segment is buried within the stem of the trimer. However, at the pH of fusion, an essentially irreversible conformational change in HA (Skehel et al., 1982; Doms et al., 1985) results in its exposure (White & Wilson, 1987). BHA becomes amphiphilic under these conditions (Doms et al., 1985), and it has been shown by

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[1] Abbreviations: BHA, bromelain-solubilized ectodomain of influenza hemagglutinin; CD, circular dichroism; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; FAB-MS, fast atom bombardment mass spectrometry; HA, influenza hemagglutinin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; LUV, large unilamellar vesicles; Mes, 2-(*N*-morpholino)ethanesulfonic acid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PC, phosphatidylcholine; SUV, small unilamellar vesicles; Tris, tris(hydroxymethyl)aminomethane.

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hydrophobic photolabeling that, at low pH, BHA interacts with liposomes through penetration of the fusion segment into the liposomal bilayer (Harter et al., 1988, 1989). In addition, from these latter studies it appeared that the fusion peptide adopts an α-helical structure upon interaction of BHA with membranes (Harter et al., 1989) and, furthermore, that the peptide is not likely to span the entire lipid bilayer (Brunner, 1989). Recently, photolabeling studies have demonstrated that the intact virus also interacts with liposomes at the pH and temperature of fusion, solely through HA2 (Brunner et al., 1991).

Because of the crucial importance of the HA2 N-terminal segment to the fusion of influenza virus, several recent studies have focused on the interaction of synthetic peptides, corresponding to the fusion segment, with lipid membranes. Murata et al. (1987) synthesized a 20-residue peptide with the sequence of the PR/8/34 strain of the virus and characterized its interaction with PC SUV. They observed peptide-induced fusion of the vesicles, assayed on the basis of lipid mixing, that exhibited a pH dependence similar to that of the fusion activity of the parent virus. On the other hand, Lear and DeGrado (1987) characterized the interaction with PC vesicles of a 20-residue peptide, corresponding to the B/Lee/40 fusion sequence, and found it to induce fusion both at neutral and acidic pH. The fusogenic capacity of the B/Lee peptide was clearly correlated with its capacity to adopt an α-helical structure upon binding to POPC SUV. A peptide corresponding to the N-terminal 16 residues also bound to vesicles but did not show the capacity to adopt an α-helix nor did it induce vesicle fusion. Interestingly, Düzgünes and Gambale (1988) observed membrane-destabilizing activity at high peptide-to-lipid ratios with HA2 N-terminal peptides as short as seven residues, although no secondary structure measurements were reported. Wharton et al. (1988) studied the lipid interactions of a number of peptides derived from HA's of the X31 strain of the virus and several variants with altered fusion activity (Gething et al., 1986). They found the pH dependence of the interaction to be influenced by the lipid composition of the vesicles used. With PC SUV, the wild-type X31 sequence exhibited fusogenic activity at both neutral and acidic pH, but with PC/cholesterol vesicles the effect of the peptide was clearly pH dependent. Also, the variant peptides were shown to have fusion properties similar to those of the corresponding entire HA's. A partial correlation between the capacity of the peptides to adopt an α-helical structure and their SUV fusogenicity was noted. More recently, Takahashi (1990) found this same correlation of α-helical structure and SUV fusion for seven different 20-residue peptides with single-residue changes in the sequence investigated by Murata et al. (1987).

In the present study, we examine the interaction of X31 peptides with POPC monolayers and bilayer vesicles. The wild-type (wt) sequence as well as two critical variant sequences E4 and E1, based on those described by Gething et al. (1986), were investigated. Rather than sonicated small unilamellar vesicles (SUV), such as those employed in the above studies, we have now used primarily larger vesicles (LUV; 0.1-μm diameter) with an essentially unstrained bilayer. The curvature of SUV membranes and their intrinsic instability appear to facilitate peptide penetration and may also provide a driving force for fusion processes that are often observed in SUV systems in the presence of hydrophobic or amphiphilic peptides (Wilschut & Hoekstra, 1986; Wilschut, 1990). Although such fusion reactions in SUV systems do reveal peptide-induced bilayer destabilization, they do not necessarily reflect the intrinsic fusogenicity of the particular peptide. The X31 wt and E4 peptides were found to interact with POPC LUV in a clearly pH-dependent manner, while the E1 mutant peptide, in which the N-terminal Gly was replaced with a Glu, like the corresponding mutant HA protein (Gething et al., 1986), showed substantially less bilayer-penetrating and destabilization activity even when bound to LUV to about the same extent as the wt peptide. In addition, the membrane-penetrating properties of the peptides were clearly correlated with their ability to adopt an α-helical structure in the presence of POPC vesicles.

Materials and Methods

Materials. Calcein was purchased from Molecular Probes, Inc. (Eugene, OR). Lipids were from Avanti Polar Lipids (Alabaster, AL), except [^{3}H]DPPC (labeled in the choline methyl groups), which was from Amersham International (Amersham, U.K.). Thesit and Triton X-100 were from Boehringer (Mannheim, FRG).

Peptide Synthesis and Purification. The peptides were synthesized as their C-terminal carboxamides by solid-phase synthesis on a Milligen 9050 continuous flow peptide synthesizer with FMOC-protected pentafluoroesters of the amino acids and PAL polystyrene resin (Milligen/Biosearch). Coupling times were 1 h, and the peptide was cleaved by treatment with trifluoroacetic acid/thioanisole/ethanedithiol/anisole (9.0:0.5:0.3:0.2) at room temperature for 2 h and then precipitated by addition of a large volume of ether. Peptides were then dissolved in DMSO and purified by reverse-phase HPLC using a preparative PRP-1 column (Hamilton) at a flow rate of 10 mL/min with a binary gradient of 20–34% B over 28 min, where B = 90% acetonitrile/10% H_2O, A = 10% acetonitrile/90% H_2O, and both buffers contained 10 mM ammonium acetate buffer, pH 6.0. Identity of the peptides was confirmed by FAB-MS and amino acid analysis. Stock solutions of peptides were made in DMSO (all peptides were soluble up to 2 mM) and stored at 0 °C for up to 2 weeks or at −20 °C for longer periods. Peptide stock solution concentrations were chosen to ensure less than 1% DMSO concentrations in the various buffers used for assays. Quantitative peptide concentrations were determined by amino acid analysis.

Surface Tension Measurements. Surface pressure was determined by the de Nöuy method (Adamson, 1976) using a Fisher Autotensiomat model 215. A POPC monolayer was spread from a chloroform solution to the desired initial surface pressure. The peptide was injected below the lipid surface, and the resulting change in surface pressure was observed. The measurements were done in buffer at pH 5.0 or 7.4 (100 mM NaCl, 5 mM sodium citrate, 5 mM Mes, 5 mM Hepes). For experiments in the presence of calcium, pH 7.4 buffer was used (30 mM $CaCl_2$, 70 mM NaCl, 5 mM sodium citrate, 5 mM Mes, 5 mM Hepes).

Tryptophan Fluorescence Measurements. The peptides all contain a Trp residue at position 14 that is highly conserved in all native HA2 sequences. The wavelength shift of the fluorescence emission maximum of this residue was used to characterize the interaction of peptide with lipid vesicles. Large unilamellar vesicles (LUV) for these experiments were prepared by freeze–thaw extrusion (Hope et al., 1985) as follows: lipid suspensions in either pH 7.4 or pH 5 buffer (100 mM NaCl, 5 mM sodium citrate, 5 mM Mes, 5 mM Hepes) were freeze–thawed 5 times by using lipid nitrogen/lukewarm water cycles and then extruded 10 times through two stacked 0.1-μm pore size polycarbonate filters (Nucleopore Corp., Pleasanton, CA) in a pressure extruder (Lipex Biomembranes Inc., Vancouver, Canada). For the preparation of small unilamellar vesicles (SUV), the lipids were hydrated in buffered solutions, followed by bath sonication (Laboratory Supplies

Inc., Hicksville, NY) under nitrogen at 20–25 °C to visual clarity. Lipid concentrations in the liposome preparations were determined by phosphate analysis, according to the method of Böttcher et al. (1961). Peptides were added to vesicle suspensions (106 μM P_i) from a concentrated stock solution in DMSO and the emission spectra were recorded (excitation wavelength 280 nm). The ratio of fluorescence intensities at 330/350 nm was determined for peptides in the presence and in the absence of LUV. The measurements were done at molar ratios of lipid to peptide of 115:1 for wt, 128:1 for E4, and 106:1 for E1. Fluorescence experiments were conducted at ambient temperature with stirring on a Spex Fluorolog model 222 with slit widths of 2 nm. Spectra (available as supplementary material) were corrected for background fluorescence, dilution, and nonlinearity of the photomultiplier response.

For binding measurements using fluorescence, peptides were diluted into buffer from concentrated DMSO stock solutions such that the final DMSO concentration was less than 1% and then titrated with LUV suspended in the same buffer. The shift in fluorescence intensity at a given wavelength can be used to quantitate the binding of the peptides to liposomes under various conditions. The fraction (R) of peptide bound, upon titration of a fixed concentration of peptide with liposomes, is determined as (Dufourcq & Faucon, 1977)

$$R = [I(c) - I(0)]/[I(s) - I(0)] \quad (1)$$

in which $I(0)$ and $I(c)$ represent the Trp fluorescence intensities at some convenient wavelength (here, 340 nm) before and after addition of liposomes to a lipid concentration c, respectively, while I(s) represents the intensity at saturation, where additional liposomes cause no further increase in fluorescence. From data thus obtained, binding constants can be determined by nonlinear least-squares fitting of data to a single-site binding equation (Lear & DeGrado, 1987):

$$[P](R - 1) - c/n - K_d + c/(nR) = 0 \quad (2)$$

where [P] and c are the peptide and lipid molar concentrations, respectively, n is the number of lipids per peptide binding site, and K_d is the dissociation constant defined for the peptide–site equilibrium. Ideally, the curve-fitting procedure provides unique K_d and n values from data of fluorescence intensity versus concentration that include the limiting parameters $I(0)$ and I(s). Here, the relatively weak binding and low peptide solubility made it difficult to determine the limiting fluorescence intensities because the high concentrations of LUV caused light scattering, which interfered with fluorescence measurements at high lipid concentrations. Thus, I(s) was also included as a variable parameter in the curve-fitting calculation (eq 1), unavoidably increasing the uncertainty in K_d and n. The values of K_d, I(s), and n were determined by unconstrained least-squares fitting of eq 2 to the data by using a personal-computer version of the program MLAB (Civilized Software, Inc., Bethesda, MD), and n was found to be 50 ± 15 as determined from several different data sets for either the wt or E4 mutant. Therefore, a constant value of 50 was used for both peptides to afford a reasonably consistent comparison of the data. It should be realized that the values of K_d and n are interdependent. For example, a change of n from 50 to 60 would change the value of K_d from 0.5 to 0.3 μM for wt peptide at pH 5.

Physical Binding Studies. POPC LUV, containing a trace of [^{3}H]DPPC, were prepared by freeze–thaw extrusion as done for Trp fluorescence measurements (see above). For these particular experiments, vesicles were prepared in pH 7.4 buffer, and the pH was adjusted to 5.0 just prior to peptide addition. The vesicles were precentrifuged for 1 h at 100000g in a Beckman TL-100 table-top ultracentrifuge (TLA 100.3 rotor). The pellet was resuspended in the same buffer and used for the binding experiments. Peptides were added (with a Hamilton syringe) from a concentrated stock solution in DMSO to 1.0 mL of a stirred POPC vesicle suspension, at the desired lipid concentration and the desired pH. The mixtures were incubated for 20 min at 25 °C and subsequently centrifuged for 2 h at 200000g. The lipid concentrations in the supernatant fractions were determined by radioactivity measurements. Less than 1% of the total radioactivity was recovered in the supernatant fractions. Conversely, in control experiments, the free peptides (i.e., in the absence of liposomes) at the concentrations used (<1 μM) were found not to sediment during the ultracentrifugation. The concentrations of peptide in the supernatants were determined by quantitative Trp fluorescence measurements, with a standard curve of known concentrations of the same peptide for calibration. Lipid and peptide in the pellets were determined by carefully removing the pellets from the vials and resuspending them in 0.3 mL of 50 mM Tris and 50 mM Thesit, pH 8.0, to dissolve the lipid. To this solution was added 0.3 mL of the NaCl, Hepes, Mes, and citrate buffer, pH 7.4. The lipid concentrations in these solubilized pellet fractions were determined by radioactivity measurements; the peptide concentrations were determined by quantitative Trp fluorescence measurements, with a standard curve of known concentrations of the same peptide in the same medium, containing a concentration of POPC equivalent to that in the samples, for calibration. The peptide-to-lipid ratio in the pellet served to calculate the total amount of vesicle-associated peptide.

Binding constants for peptide-to-vesicle association were calculated from the amount of peptide in the supernatant (P_{sup}) and the corresponding amount in the pellet (P_{pel}). The fraction bound (R) was determined, taking the sum of P_{sup} and P_{pel} as the total (P_{tot}). Only those measurements were considered where P_{tot} was more than 70% of the initial amount of peptide.

Leakage of Liposome Contents. The leakage of liposome contents to the external medium was kinetically monitored by the release of calcein trapped inside the vesicles (Straubinger et al., 1983). This particular fluorescent probe was used because it shows little variation in quantum yield in the pH range studied. The sodium salt of calcein (40 mM) was encapsulated in POPC liposomes, buffered with 10 mM Hepes at pH 7.4. At this concentration fluorescence self-quenching occurs. LUV for these experiments were prepared by reverse-phase evaporation in buffer, followed by single extrusion through one 0.1-μm pore size polycarbonate filters (Wilschut et al., 1980). Nontrapped calcein was removed by gel chromatography on Sephadex G-50. Vesicles were eluted with 100 mM NaCl, 5 mM sodium citrate, 5 mM Mes, and 5 mM Hepes, pH 7.4. As for all other experiments in this work, lipid concentrations in the vesicle suspensions were determined by phosphorus assay according to the method of Böttcher et al. (1961). Upon addition of peptides from a DMSO stock solution to a vesicle suspension adjusted to the appropriate pH, leakage of calcein to the external medium was followed by the increase in fluorescence (caused by calcein dilution and consequent relief of self-quenching) with excitation and emission wavelengths of 490 and 520 nm, respectively. 100% leakage was established by lysing the vesicles with 0.5% (w/v) Triton X-100. Fluorescence measurements for these experiments were done using a SLM-8000 spectrofluorometer with slit widths of 4 nm.

Circular Dichroism. Spectra were recorded on an Aviv model 62 DS circular dichroism spectrophotometer at room

wt G L F G A I A G F I E N G W E G M I D G - $CONH_2$

E4 G L F E A I A G F I E N G W E G M I D G - $CONH_2$

E1 E L F G A I A G F I E N G W E G M I D G - $CONH_2$

FIGURE 1: Amino acid sequences of peptides representing the N-terminus of the HA2 chain of the hemagglutinin of the X31 strain of influenza (carrying the Hong Kong HA).

temperature with a 5-mm cell. All spectra were baseline corrected and smoothed. Peptides were incorporated into POPC vesicles (SUV) at a lipid-to-peptide ratio of 200:1 as follows. Peptides in 1,1,1,3,3,3-hexafluoro-2-propanol were mixed with POPC in chloroform and dried to a film with a stream of nitrogen. The residue was vacuum dried at 100 mTorr overnight and then hydrated. For measurements to be done at pH 5.0, the lipid–peptide mixture was hydrated with water at pH 5 (adjusted with dilute HCl; the acidic peptide itself serves as a buffer at this pH). For pH 7.4 measurements, 10 mM ammonium acetate at pH 7.4 was used to hydrate the lipid–peptide mixture. Hydration was followed by sonication under argon to visual clarity with the bath sonicator. Final peptide concentrations in the samples were 10 μM. We chose this method of premixing the peptide with the lipid using the fluorinated cosolvent in order to avoid unwanted UV absorption by DMSO. The lipid-to-peptide ratio was kept large to ensure that the peptide was fully bound to the vesicles, and SUV were used to minimize light-scattering artifacts.

Results

Peptide Synthesis. In this study, we investigate the lipid interactions of 20-residue synthetic peptides (Figure 1) corresponding to previously studied (Gething et al., 1986) "E1" and "E4" Gly-to-Glu substitutions in the N-terminus of the HA2 subunit of influenza virus hemagglutinin. The peptides were synthesized by using FMOC-protected amino acids, eliminating the problems with aspartamide formation at Asp-Gly sequences (Bodansky & Martinez, 1983) that we had encountered in earlier syntheses using Boc-protected amino acids. HF was used for the final deprotection step. Although pyroglutamic acid formation (from N-terminal glutamic acid cyclization) was expected to cause problems during purification of the E1 peptide, this side reaction was not observed when the peptide was purified by reverse-phase HPLC at pH 6.0, and the peptide was promptly frozen and lyophilized. The peptides appeared to be stable when stored as lyophilized powders at −5 °C.

Insertion of Peptides into Lipid Monolayers. We studied the interaction of the HA peptides with phospholipid monolayers, a model system chosen to simulate one side of a bilayer structure. Surface-active peptides bind to phospholipid monolayers, frequently causing an increase in the surface pressure of the monolayer (if the total area of the monolayer is kept constant). When the increase in surface pressure is significant, it is generally believed that the hydrophobic portion of the peptides penetrates into the fatty acid region of the lipid monolayer (Phillips & Sparks, 1980). A widely used measure of the affinity of a peptide for a surface is the critical pressure of insertion (Π_c), the surface pressure above which a peptide is unable to penetrate into a lipid monolayer (Verger & Pattus, 1982). Figure 2 illustrates the increase in surface pressure ($\Delta\Pi$) associated with the addition of the wt and E1 peptides to a monolayer of POPC as a function of the initial monolayer surface pressure (Π). As has been previously observed for other peptides [e.g., Briggs et al. (1985)], $\Delta\Pi$ is a linear

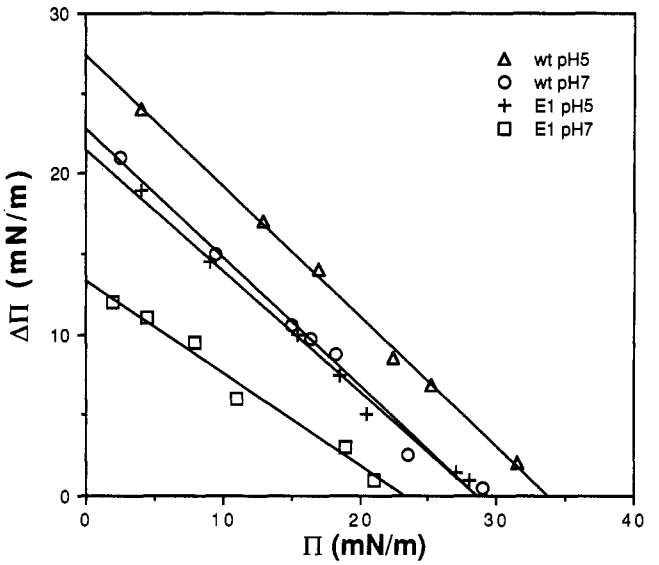

FIGURE 2: Data showing the increase in surface pressure ($\Delta\Pi$) of lipid monolayers as a function of initial surface pressure (Π). Peptides were injected into buffer solutions (see Materials and Methods) below the lipid surface to a final concentration of 2 μM for wt and 3 μM for E1. Symbols: (circles) wt; (squares) E1 at pH 7.4; (triangles) wt; (crosses) E1 at pH 5.0. Π_c, the critical pressure of insertion, is defined as the lipid monolayer pressure at which no further increase occurs upon addition of peptide to the subphase solution. Similar plots were used to determine the results in Table I.

Table I: Critical Pressure of Insertion for HA Peptides

	critical pressure of insertion into POPC monolayers (mN/m)			equilibrium spreading pressure of peptide alone (mN/m)		
peptide	pH 7.4	pH 5.0	pH 7.4[a]	pH 7.4	pH 5.0	pH 7.4[a]
wt	28	33	32	23	25	25
E4	22	33	28	20	26	24
E1	23	28	25	14	20	18

[a] $[Ca^{2+}]$ = 30 mM.

function of Π. Extrapolation to zero $\Delta\Pi$ gives the critical surface pressure (Π_c) for the peptide's insertion into POPC monolayers. At pH 7.4, the critical surface pressure is 28 mN/m. This value is above the equilibrium spreading surface pressure of wt peptide under these conditions (23 mN/m, Table I), which indicates that the surface pressure increase observed with lipid monolayers represents favorable peptide/lipid interactions. For comparison, melittin, a membrane-active peptide toxin from bee venom, has a critical surface pressure of insertion of 40 mN/m for lecithin/dicetyl phosphate/cholesterol (7:2:1) monolayers [calculated from data of Sessa et al. (1969); lecithin alone was said to give essentially the same result], and functional signal peptides have Π_c on the order of 28 mN/m for egg phosphatidylethanolamine/phosphatidylglycerol monolayers (Briggs et al., 1985).

Referring again to Table I, the critical surface pressure of insertion for wt peptide is increased markedly from 28 mN/m at pH 7.4 to 33 mN/m at pH 5.0. The E4 peptide showed an even larger pH dependence of its critical surface pressure, with a value identical with that of the wt peptide at pH 5.0 (Π_c = 33 mN/m) but a strikingly lower critical pressure of insertion at pH 7.4 (Π_c = 22 mN/m). In contrast, the E1 peptide had lower values of Π_c at both pH values, in keeping with its inability to function in the intact protein in vitro. Thus, the functional peptides at pH 5.0 show values of Π_c greater than 30 mN/m, whereas the nonfunctional E1 peptide (and wt and E4 at pH 7.4) have Π_c below this value. The difference between the highest value of Π_c for the nonfunctional E1 at

Table II: Fluorescence Intensities (R^a 330/350 nm) and λ_{max} (nm) for HA Peptides[b]

peptide	no liposomes		+POPC LUV		+POPC SUV
	pH 7.4	pH 5.0	pH 7.4	pH 5.0	pH 5.0
wt	0.74 (349)	0.74 (349)	0.77 (347)	1.39 (330)	1.60 (329)
E4	0.74 (349)	0.73 (349)	0.78 (347)	1.48 (330)	1.60 (329)
E1	0.73 (349)	0.72 (349)	0.75 (349)	0.90 (345)	1.40 (332)

[a] R, the ratio of fluorescence intensity at 330 and 350 nm, was used as an index for the environment of tryptophan residues. [b] Peptides (in DMSO) were added into the buffer or buffer containing POPC LUV (106 μM). λ_{max} was measured at a lipid-to-peptide ratio of 100. λ_{ex} was 280 nm.

pH 5.0 (28 mN/m) and functional wt at pH 5.0 (33 mN/m) is substantial; 5 mN/m corresponds to a surface free energy difference of 3.6 kJ/mol (0.86 kcal/mol) if one conservatively estimates the inserted area of these peptides to be about 120 Å^2/molecule (Briggs et al., 1985).

The increased affinity of the peptides for bilayers at pH 5.0 could be a consequence of a decrease of their free energy of transfer from water to the less polar bilayer interior (Von Heinje & Blomberg, 1979) due to partial protonation of the Glu side chains. However, an equally, perhaps even more, important contribution could come from the increased α-helix stability associated with neutralization of the negatively charged Glu side chains; these would be in close proximity on one side of the α-helix when the peptide binds to bilayers in an α-helical conformation. If this effect were significant, then divalent cations such as Ca^{2+} should have an effect similar to low pH. The reason is that Ca^{2+} would be expected to bind electrostatically to the peptide carboxylates [Ca^{2+} does not bind appreciably to the zwitterionic lipid used to form the bilayers (McLaughlin et al., 1978)] but not to enter the hydrophobic portions of the bilayer. In fact, 30 mM $CaCl_2$ added to the pH 7.4 buffer was found to partially mimic the effect of lowering the pH on the critical insertion pressures (Table I).

Peptide–Liposome Interaction: Tryptophan Fluorescence Studies. The binding of the peptides to POPC vesicles was followed by measuring the fluorescence of its Trp residue at position 14. An increase in the hydrophobicity of the peptide's environment will result in a blue-shift of the Trp fluorescence emission (Lakowicz, 1983), thus providing a sensitive assay for the capacity of the peptides to penetrate into the hydrophobic core of a lipid bilayer membrane. Data determined from fluorescence spectra for the wt, E4, and E1 peptides at pH 5.0 and 7.4 in the absence or presence of POPC LUV and SUV are shown in Table II. The emission maximum of these peptides in the absence of lipid vesicles is 349 nm, consistent with the Trp being largely exposed to solvent. In the presence of vesicles the wt and E4 peptides show a large shift to 330 nm, which is consistent with a transfer to a more hydrophobic environment. In contrast, the E1 peptide showed a much smaller shift to 345 nm. In the presence of small unilamellar POPC vesicles (SUV), the wt and E4 peptides showed changes in emission maxima similar to those observed with LUV, but the E1 peptide showed a much greater change in emission maxima with SUV than LUV. This is consistent with the view (Wilschut & Hoekstra, 1986; Wilschut, 1990) that SUV have a higher potential to be fused by and interact with hydrophobic peptides due to the small radius of curvature of the vesicular surface. Thus, it is not surprising that, in the previous study of Wharton et al. (1988), SUV were less able to discriminate between the functional (wt and E4) and nonfunctional (E1) peptides.

The ability of the peptides to interact with LUV in our study depended markedly on pH (Table II). At pH 7.4, a very small change in wt Trp emission maxima accompanied addition of LUV, whereas a much larger effect was observed at pH 5.0. Also, with both wt and E4, but not with E1, Trp emission

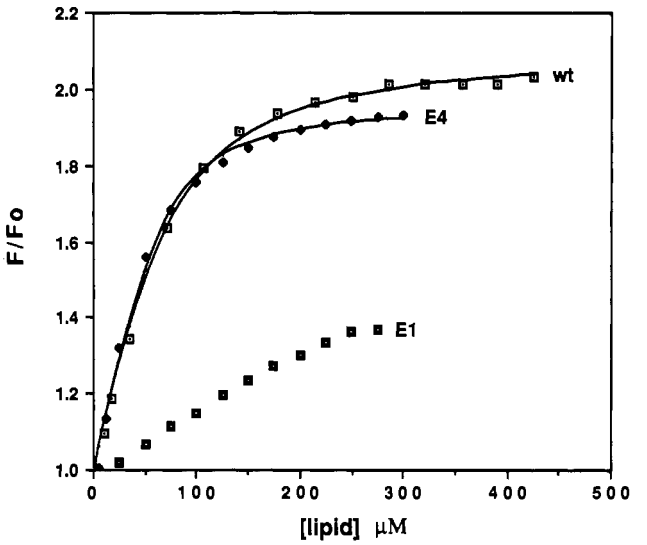

FIGURE 3: Titration of X31 peptides with POPC LUV as monitored by fluorescence. Fluorescence intensities at 340 nm (F) were measured for each peptide at 1.3 μM in pH 5.0 buffer with increasing amounts of added POPC LUV. Intensity ratios (F/F_0) are plotted where F_0 is the fluorescence intensity measured in the absence of lipid. Solid lines are calculated curves for a single site binding equilibrium isotherm fit to the experimental data with the program MLAB (Civilized Software, Inc., Bethesda, MD) with an assumed binding site size of 50 lipids/peptide. K_d's so determined were 0.5 μM for wt and 0.2 μM for E4.

maxima in the presence of LUV at pH 7.4 were shifted to shorter wavelength by 30 mM Ca^{2+} (data not shown), consistent with the effect observed with monolayer insertion experiments (cf. above).

As a measure of peptide binding to bilayers, the ratio of the fluorescence intensities at 330 and 350 nm (reflecting both the blue-shift and altered quantum yield of the Trp fluorescence) has also been used (Wharton et al., 1988). The I_{330}/I_{350} ratios for the peptides (Table II) changed in parallel with the shifts in emission maxima as a function of pH and the addition of vesicles. These fluorescence data, together with the results of the monolayer penetration experiments, show that the E4 and wt peptides penetrate into phospholipid membranes more at pH 5.0 than at pH 7.4. The E1 peptide showed, by all measures, the least penetration of the three peptides at both pH values.

Peptide Binding Isotherms. Figure 3 illustrates the change in fluorescence intensity at 340 nm when 1.3 μM of each peptide at pH 5.0 is titrated at ambient temperature with increasing concentrations of LUV. The data for wt and E4 can be described by a simple binding isotherm with a limiting stoichiometry of 50 lipids/peptide and dissociation constants of 0.5 μM for wt and 0.2 μM for E4. In contrast, E1 showed a much smaller increase in intensity, indicating significantly less binding. A reliable estimate of the binding stoichiometry and affinity could not be obtained for this peptide by this method because of the relatively small change in emission intensity and also because this peptide caused aggregation of

Table III: Peptide–Lipid Vesicle Binding Measured by Physical Separation

peptide	pH	[lipid] (μM)	P_{sup}	P_{pel} (nmol)	P_{tot}	recovery (%)	R	K_d[a] (μM)
wt	7.4	50	0.39	0.22	0.61	79	0.36	1.4
wt	5.0	50	0.28	0.26	0.54	70	0.48	0.8
E1	5.0	50	0.51	0.11	0.62	78	0.18	4.1

[a] All K_d's calculated for a site size of 50 lipids/peptide.

the vesicles and associated light-scattering artifacts.

In order to provide a more direct comparison of the binding affinities of the different peptides for vesicles, experiments were done in which vesicle-bound peptide was physically separated from unbound peptide by centrifugation. Quantitation of the peptide in solution and in the pellet allows calculation of an apparent K_d (Table III), as described under Materials and Methods. The value of the K_d calculated by this method for the wt peptide at pH 5.0 (0.8 μM) is in reasonable agreement with that calculated by fluorescence titration (0.5 μM).[2] This method also allows the estimation of the K_d for the wt peptide at pH 7.4. The affinity of this peptide for vesicles is only about 2-fold less at pH 7.4 than at pH 5.0, even though binding at the higher pH gives very little shift in emission wavelength of the peptide tryptophan. Binding of the E1 peptide at pH 5.0, although even lower than that of the wt at pH 7.4, was still significant, but again, very little shift in the Trp fluorescence was observed. Since Trp fluorescence shifts are generally interpreted as a measure of how deeply the peptide penetrates into the bilayer, these results again suggest that penetration of peptide into the bilayer interior is more enhanced by low pH for wt and E4 than for E1 and that binding and penetration are, to some extent, independent.

Circular Dichroism (CD) Spectroscopy. In aqueous solution in the absence of phospholipid vesicles, the peptides displayed CD spectra corresponding to a random coil conformation, with an ellipticity at 222 nm [θ_{222}] of approximately −1500 deg cm^2 dmol^{-1}. Their spectra (Figure 4) were then measured at pH 7.4 and 5.0 in the presence of SUV (small vesicles minimize light-scattering artifacts) in sufficient excess to bind essentially all the peptide (see the legend to Figure 4 for details). At pH 7.4, all three peptides displayed spectra that are consistent with the formation of a partially helical structure, with the E1 peptide showing the smallest extent of helix formation. The CD minima at 222 and 208 nm (diagnostic of α-helix formation) for the wt and E4 peptide were considerably greater at pH 5 than at neutral pH. Analysis using the method of Greenfield and Fasman (1969) indicated that these two peptides were approximately two-thirds α-helical under these conditions. In contrast, the CD minima for the E1 peptide were considerably smaller than observed for wt and E4 (all at pH 5.0), and the E1 spectrum was different. The shape of the spectrum suggests partial β-structure. IR spectra of E1 in lipid multilayers determined according to methods described by Rafalski et al. (1990) also indicated β-structure (data not shown). Thus, the conformations of all three peptides

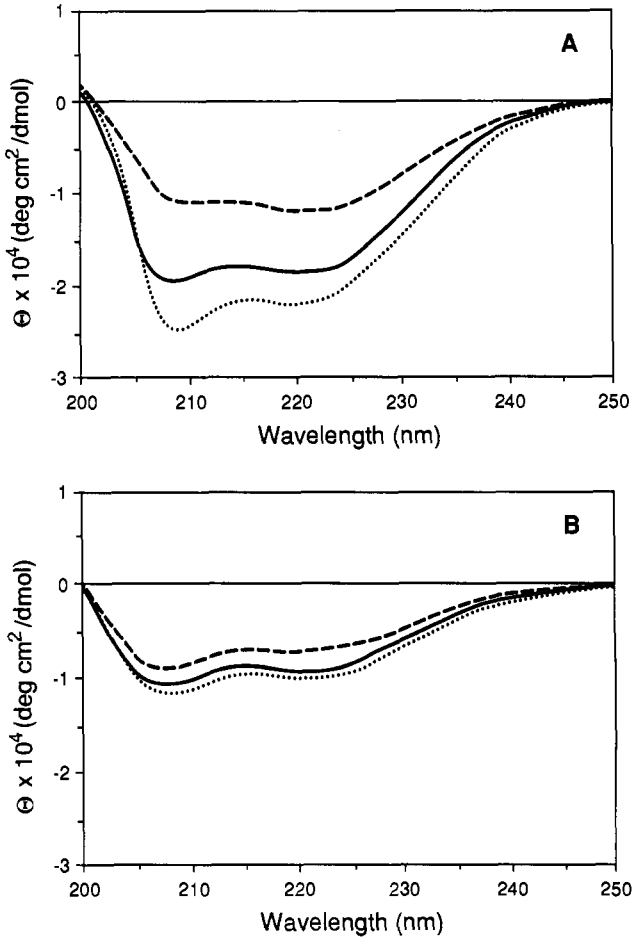

FIGURE 4: CD spectra of X31 peptides (10 μM) incorporated into POPC SUV bilayers at a lipid-to-peptide molar ratio of 200:1. (Solid line) wt; (dotted line) E4; (dashed line) E1. (A) pH 5.0; (B) pH 7.4.

appeared to be sensitive to pH, but only the E4 and wt peptides were predominantly α-helical in vesicles at acid pH. The slightly higher ellipticity of the E4 peptide relative to the wt may be accounted for by the internal glutamic acid residue replacing the helix-destabilizing glycine at position 4.

Peptide-Induced Leakage of Liposomal Contents. The addition of the peptides to LUV produced membrane destabilization as measured by the leakage of calcein from the vesicles. Figure 5A illustrates the time course for the release of calcein from LUV (lipid concentration 50 μM), induced by addition of the wt, E4, or E1 peptides. At the same lipid and peptide concentrations, all three peptides caused a measurable rate of leakage: the wt peptide was the most potent, the E4 peptide was one-fourth as active as the wt, and E1 had about one-twentieth the activity of the wt. The leakage rate was sensitive to the peptide-to-lipid ratio. Figure 5B illustrates the dependence of the initial rate of leakage on the concentrations of the three peptides at constant (50 μM) lipid concentration. Under these conditions, the initial rates were approximately linear with respect to peptide concentration, and the relative activity of the peptides was maintained.

To determine if the differences in leakage-induction potency of the peptides might be due to differences in binding affinities,

[2] One possible explanation for the difference between K_d values determined by physical separation versus fluorescence titration is that, in the physical separation method, at least 20% of the peptide was always observed in solution, even at saturating lipid concentrations where all the peptide should be bound, on the basis of the value of K_d determined fluorimetrically. This suggests that there is a small population of the peptide that is unable to bind to vesicles, possibly due to the formation of aggregates that dissociate slowly under our experimental conditions. Such a possibility seems reasonable in light of the hydrophobic nature of the peptides. If such a population exists, it would not be detected by fluorescence titration (which only measures a relative change in fluorescence intensity), but it would be detected and considered as free peptide by the physical separation method.

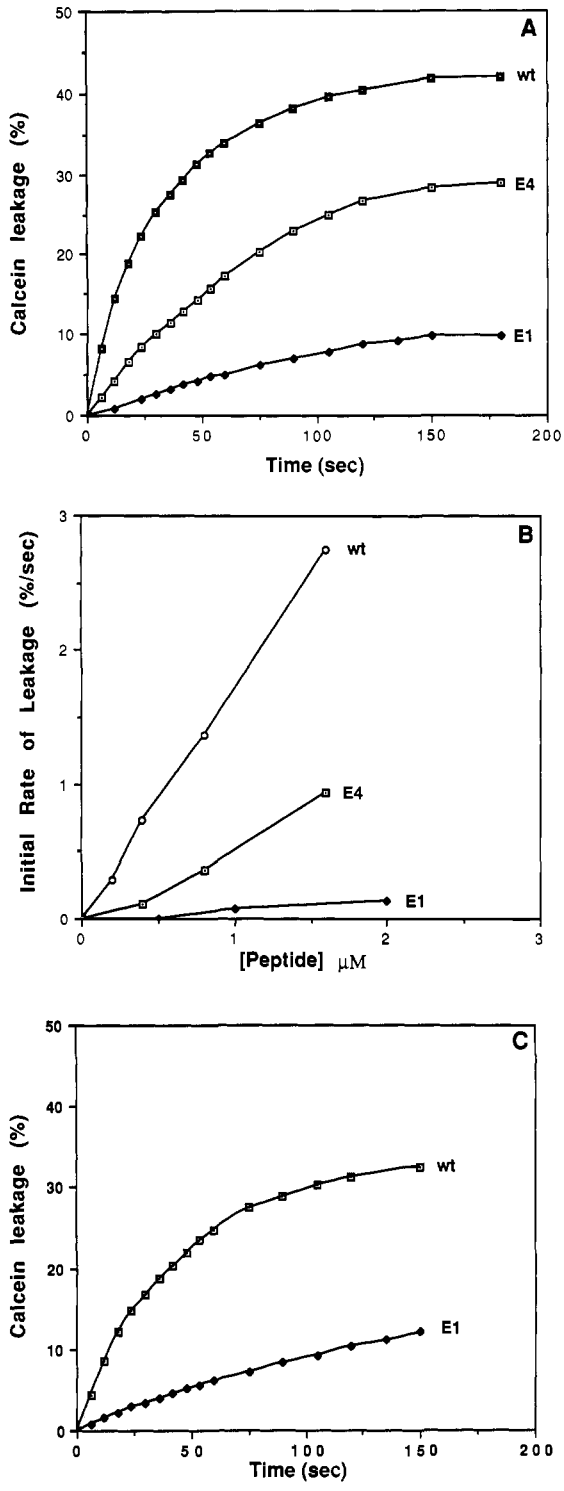

FIGURE 5: Leakage of liposomal contents induced by X31 peptides. (A) Time course of the peptide-induced release of calcein entrapped in POPC LUV. Vesicles (50 μM P_i) were suspended in pH 5.0 buffer, and peptides were added at final concentration of 0.8 μM wt, 0.8 μM E4, and 1.0 μM E1. 100% leakage was established by lysing the vesicles with Triton X-100 (0.5% w/v). Data points were taken from continuous records and are shown to facilitate discrimination between curves. (B) Initial rate of calcein leakage from POPC LUV in buffer at pH 5.0 as a function of peptide concentration. The lipid concentration was the same as above. (C) Calcein leakage at pH 5.0 from 50 μM POPC LUV induced by 0.4 μM wt and 1.6 μM E1 peptides. These concentrations were chosen to give approximately equal percentages of vesicle binding site saturation by each peptide. Calculated values were 20% for wt and 25% for E1 on the basis of the binding parameters calculated from physical separation experiments (see Table III). These values correspond to molar ratios of lipid to bound peptide of 250:1 for the wt and 200:1 for E1. Again, data points were taken from continuous records.

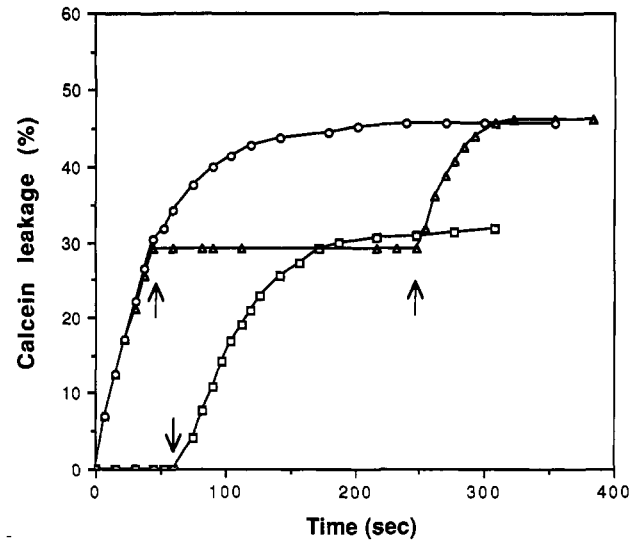

FIGURE 6: pH dependence of the X31-wt-peptide-induced leakage of liposomal contents. The time course of the wt-peptide-induced leakage of calcein entrapped at 40 mM in POPC LUV (50 μM lipid) was monitored with time following addition of peptide to a final concentration of 0.77 μM. Data shown are for three separate experiments: (circles) pH constant at 5; (squares) pH changed at the times denoted by the arrows from 7 to 5; and (triangles) pH changed from 5 to 7, then back to 5. Data points were taken from continuous records. The flat portion of the curve representing the reacidification experiment was corrected for the gradual decrease in fluorescence intensity observed upon pH change in a solution of free calcein.

vesicle contents leakage rates were determined for the wt and E1 peptides by using peptide concentrations calculated (based on the binding parameters measured by the physical separation technique) to give approximately equal peptide-to-lipid ratios at the vesicle surface. The result is shown in Figure 5C. The E1 peptide still caused a significantly lower rate of leakage, consistent with the previously discussed differences in monolayer and bilayer penetration.

The wt peptide-induced leakage of calcein depended strongly on pH and did not occur appreciably at pH 7. As shown in Figure 6, when peptide was added to vesicles at pH 5.0 and the pH was then shifted to 7.0 prior to completion of leakage, the leakage of calcein abruptly ceased. Changing the pH back to 5.0 caused the leakage to resume, and it appeared to reach the same leakage level as when the pH of the vesicles was not changed in the middle of the experiment. Similarly, if peptide was added to vesicles at pH 7.0, no leakage was observed until the pH was lowered to 5.0.

Discussion

It is well established that the N-terminus of the HA2 subunit of the influenza virus hemagglutinin plays a crucial role in the membrane fusion activity of the virus. At neutral pH, the 20-residue segment is buried in the stem of the spike trimer (Wilson et al., 1981; Wiley & Skehel, 1987), but at the low pH within the endosomes it is exposed, due to a conformational change in the molecule (Skehel et al., 1982; Doms et al., 1985; White & Wilson, 1987). Although the subject of some debate (Ellens et al., 1990), the prevailing view on the subsequent action of this fusion segment in mediating the fusion process between the viral envelope and the endosomal target membrane involves its penetration into the target membrane (Doms et al., 1990; Stegmann et al., 1987b, 1989, 1990; Harter et al., 1988, 1989; Brunner et al., 1991).

In the present study, we have established a functional correlation between synthetic peptides and previously studied mutants of the influenza HA protein derived from the A/Japan/305/57 strain of the virus (Gething et al., 1986). The

wild-type and E4 mutant HA proteins, when expressed in transfected cells, were fusogenic only at acidic pH, and the corresponding synthetic peptides show a strong pH dependence to their conformational and membrane-penetrating properties. Further, the fusion activity of the E1 mutant HA protein was almost completely abolished: cell–cell fusion was unobservable and fusion of erythrocytes to the HA-expressing cells (measured by a very sensitive assay) was greatly impaired. The corresponding synthetic peptide has a considerably reduced ability to penetrate into phospholipid membranes, form α-helices, and cause leakage from liposomes, at both pH 7.4 and 5.0. The ability of the peptides to disrupt membranes is not a simple consequence of their hydrophobicities (E1 and E4 are equally hydrophobic), nor of their binding to bilayers, but instead appears to require the ability to form α-helical bilayer-penetrating structures.

Our present results help to resolve apparently discordant reports in the literature concerning the pH dependence of the membrane-penetrating properties of various HA2-derived peptides. Murata et al. (1987) have shown that a 20-residue peptide from the N-terminus of HA2 from the A/PR/8/34 strain of influenza causes fusion of POPC SUV in a strongly pH-dependent manner. On the other hand, a 20-residue peptide corresponding to the N-terminus of the B/Lee strain causes fusion of POPC SUV at neutral pH as well as at pH 5.0 (Lear & DeGrado, 1987). Wharton et al. (1988) found that 23- and 20-residue HA2 N-terminal peptides representing the X31 strain fuse SUV composed of POPC/cholesterol (1:1) in a strictly pH-dependent manner, whereas POPC SUV were found to fuse at both pH 5 and 7. This might be accounted for by the reduced curvature (larger diameter) expected for cholesterol-containing sonicated SUV (Johnson, 1973; Lelkes, 1984).

The reported differences among HA-derived peptides in pH-dependent membrane-interaction can be explained by considering the sequences of the individual peptides:

A/PR/8/34	H_2N–GLFGAIAGFIEGGWTGMIDG–CO_2H
X31	H_2N–GLFGAIAGFIENGWEGMIDG–$CONH_2$
B/Lee	H_2N–GFFGAIAGFLEGGWEGMIAG–$CONH_2$

A major difference between these peptides is the number of ionizable groups within the last four C-terminal residues. The A/PR/8/34 peptide used by Murata et al. (1987) contains two: an aspartic acid and the α-carboxylate group. The X31 wt has only one due to the C-terminal amidation, and the B/Lee peptide (Lear & DeGrado, 1987) has the uncharged helix-promoting alanine. Negatively charged groups within the last turn of an α-helix, particularly terminal carboxylates, strongly destabilize this conformation due to unfavorable electrostatic interactions with the helical macrodipole (Shoemaker et al., 1987). Thus, if α-helix formation is essential for membrane penetration and perturbation (as this work indicates), one would expect that the X31 and A/PR/8/34 peptides would be active only at acid pH and that the B/Lee could be active also at neutral pH.

Another critical finding of the current paper is the importance of using LUV to characterize peptide/lipid interactions rather than SUV. Earlier work with HA2-derived peptides was conducted with SUV, which have a smaller radius of curvature and are intrinsically less stable than LUV. Thus, SUV are less able to discriminate between peptides from functional versus nonfunctional mutant proteins. For instance, the X31 E1 peptide is known to be capable of penetrating into POPC SUV at low pH on the basis of fluorescence studies (Table II above; Wharton et al., 1988), and the wt and E1 peptides are also known to penetrate POPC SUV at neutral pH on the basis of this assay. Addition of cholesterol in the study of Wharton et al. (1988) increased the discrimination among the peptides by stabilizing the SUV, most likely by making them larger (cf. above). Interestingly, this effect has not been observed with LUV. Although, in agreement with well-documented effects of cholesterol on the stability and permeability of PC bilayers (Demel & De Kruijff, 1976), bilayer penetration and the initial rates of calcein leakage were substantially reduced in the presence of cholesterol; importantly, POPC and POPC/cholesterol vesicles of 0.1-μm diameter showed equivalent pH dependences for vesicle destabilization and no evidence whatsoever for vesicle membrane fusion in the presence of the X31 wt peptide (J. Wilschut et al., unpublished results). Therefore, while peptide-induced SUV fusion does correlate with the ability of the peptides to penetrate into and destabilize lipid bilayers, the actual fusion reaction seen in SUV systems is likely to require the high degree of curvature of the SUV bilayer. Accordingly, a causal relationship between peptide-induced liposome fusion and leakage of aqueous liposome contents, as proposed by Wharton et al. (1988) on the basis of experiments with SUV, is unlikely. Our present results with LUV do show, however, a strong correlation between peptide penetration and bilayer destabilization as assessed by leakage of liposome contents. Since these peptides, in the context of viral HA, are strongly implicated in fusion, a role for peptide-induced bilayer destabilization in fusion is certainly suggested.[3]

In summary, binding of the X31 peptides to lipid bilayers depends on pH, the extent of binding being limited at neutral pH and significant at acid pH for all three peptides studied. This is likely to be related to the ionization state of the glutamic acid residues present on each of the peptides. On the other hand, even at the same degree of binding, there are differences in the ability of the peptides to cause vesicle membrane destabilization. These appear to correlate with the peptides' critical pressures of insertion into lipid monolayers and with their adopting an α-helical structure upon interaction with lipid bilayers. The association of α-helical structure with penetration is quite consistent with the view that the free energy of transfer of peptide amide groups from water into the apolar bilayer interior is significantly reduced by the intrapeptide hydrogen-bonding pattern of an α-helix. The sensitivity of vesicle destabilization to the state of ionization and the position of the acidic Glu residues in the sequence suggests that the depth of penetration of peptide into the bilayer structure is an important parameter determining bilayer destabilization, and the progressively lower destabilization observed for the E4 and E1 mutants suggests that penetration of the N-terminus is important. The model peptide "GALA" (Parente et al., 1988) shows similar pH- and sequence-dependent vesicle destabilization, again consistent with pene-

[3] Regarding the issue of pure-peptide-induced fusion of LUV, many requirements beyond bilayer destabilization have to be met in order for efficient fusion to occur (Wilschut, 1990). One such requirement envisioned for the merging of two continuous bilayer structures is stabilization of the geometrically necessary region of high intrinsic curvature of the lipid–water interface in the fusion intermediate (Gruner, 1985; Gruner et al., 1985). Preliminary observations (J. Wilschut et al., unpublished results) indicate that the X31 wt peptide, but not E1, induces extensive fusion of DOPE/DOPC/cholesterol (1:1:1) LUV in a pH-dependent manner. This result suggests that the HA-derived wt peptide may itself be capable of altering the intrinsic curvature parameter of the vesicle bilayer in a manner favoring fusion, possibly through the formation of inverted micellar structures serving as transient fusion intermediates (Siegel, 1987; Bentz & Ellens, 1988).

tration of peptide α-helices into the bilayer. Düzgünes and Gambale (1988) have observed lipid vesicle and planar bilayer destabilization at neutral pH induced by short seven-residue peptides corresponding to the N-terminus of the X31 wt peptide and the E1 as well as the E4 mutant. These authors also found a reduced membrane-penetrating activity for the E1 peptide. However, in this study, correlation between bilayer penetration and peptide structure was not investigated. Furthermore, extremely high peptide concentrations were used: in the vesicle destabilization experiments, these amounted to an approximately 1:1 peptide-to-lipid mole ratio, while the leakage rates were low compared to those observed in the present study.

The accumulated results of many studies with the HA2 N-terminal peptides make it clear that they interact with lipid bilayers in a manner consistent with their often-noted hydrophobic amphiphilic character. The finding of a pH dependence for HA2 N-terminal peptide membrane penetration and perturbation suggests that the entire HA molecule exhibits two levels of pH control in the expression of its fusion activity, the first involving the pH-dependent conformational change and the second being at the level of the interaction of the fusion segment with the target membrane. Several pH-dependent events, each with a pK_a near 5.0, would produce a highly cooperative process, explaining why the steepness of the pH dependence of hemolysis and fusion observed for the intact virus is beyond that expected for a simple titration curve (Daniels et al., 1985, Doms et al., 1985). Whether or not this is the case, it is important to emphasize that the bilayer-destabilizing properties of the fusion segment of HA2 peptides are most likely only one aspect, albeit an essential one, of the processes involved in biologically relevant fusion of influenza virus. A full understanding of those processes will undoubtedly require a more detailed structural knowledge of the membrane interactions of the entire hemagglutinin.

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Supplementary Material Available

Six figures showing the fluorescence spectra of X31 peptides in the presence and absence of POPC LUV or SUV in pH 5.0 buffer (7 pages). Ordering information is given on any current masthead page.

Registry No. wt, 136006-27-4; E1, 136006-28-5; E4, 136033-71-1; POPC, 26853-31-6; H^+, 12408-02-5.

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Breakdown of the Photosystem II Reaction Center D1 Protein under Photoinhibitory Conditions: Identification and Localization of the C-Terminal Degradation Products

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ABSTRACT: Illumination of a suspension of thylakoids with light at high intensity causes inhibition of the photosystem II electron transport activity and loss from the membrane of the D1 protein of the photosystem II reaction center. Impairment of the electron transport activity and depletion of D1 protein from the thylakoid membrane of pea were investigated with reference to the presence or absence of oxygen in the suspension. The breakdown products of the D1 protein were identified by immunoblotting with anti-D1 polyclonal antibodies which were proven to recognize mainly the C-terminal region of the protein. The results obtained show that (i) the light-induced inactivation of the photosystem II electron transport activity under anaerobic conditions is faster than in the presence of oxygen; (ii) depletion of D1 protein is observed on a longer time scale with respect to loss of electron transport activity and is faster when photoinhibition is performed in the presence of oxygen; (iii) C-terminal fragments of D1 are only observed when photoinhibition is carried out anaerobically and are mainly localized in the stroma-exposed regions; and (iv) the fragments observed after anaerobic photoinhibition are quickly degraded on further illumination of the thylakoid suspension in the presence of oxygen.

Light-induced inhibition of oxygenic photosynthesis is a widely documented phenomenon (Powles, 1984). At the molecular level, the target of photoinhibition is photosystem II (PSII),[1] whose electron transport activity is severely impaired by high-intensity illumination. In the PSII reaction center all the redox components necessary for the primary photochemical events are associated with the heterodimer formed by the two proteins D1 and D2 (Nanba & Satoh, 1987; Barber et al., 1987). The D1 protein plays a special role in the metabolic machinery of PSII, being characterized by a turnover higher than that of any other thylakoid polypeptide (Mattoo et al., 1981). Moreover, its degradation rate increases

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[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenolindophenol; LHCP, the main light harvesting complex serving photosystem II; PAGE, polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; pheo, pheophytin; PS, photosystem; P_{680}, primary electron donor in PSII; Q_A, first quinone acceptor in PSII; Q_B, second quinone acceptor in PSII; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tyr_Z, tyrosine 161 on D1 protein.

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